THE IMPORTANCE OF RIBOFLAVIN, PANTOTHENIC ACID, NIACIN AND PYRIDOXINE IN THE NUTRITION OF FOXES [1]

A. E. SCHAEFER, C. K. WHITEHAIR AND C. A. ELVEHJEM
Department of Biochemistry and Veterinary Science, University of Wisconsin, Madison

TWO FIGURES

(Received for publication April 30, 1947)

The nutritional requirements of the fox have been under investigation in our laboratory in which purified casein-sucrose rations supplemented with synthetic vitamins have been used. The requirements for folic acid and another unidentified factor or factors in fresh liver have been reported (Schaefer et al., '47). Since fox pups receiving the purified ration supplemented with all the known synthetic vitamins grow as well as fox pups receiving a stock meat, cereal, and liver ration for a period of 14–16 weeks, it is possible to study the requirement for these vitamins if the deficiencies develop within 12 to 14 weeks after being placed on the deficient ration. Experiments were initiated, therefore, in which each of the following vitamins was omitted from the complete basal ration: riboflavin, pantothenic acid, niacin or pyridoxine.

EXPERIMENTAL

Weanling silver and red fox pups 6 to 7 weeks of age were used in these studies. The general experimental procedure has been described previously (Schaefer et al., '47). The

[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station, Project 614, Fur Farm Research.

Copyright, 1947
The Wistar Institute of Anatomy and Biology.
All rights reserved.

basal ration had the following composition: sucrose 66%, casein (Smaco Vit. Test) 19%, cottonseed oil 8%, cod liver oil 3% and salts IV (Phillips and Hart, '35) 4%. Each 100 gm of ration was supplemented with 0.2 mg thiamine chloride, 0.2 mg pyridoxine hydrochloride, 0.4 mg riboflavin, 1.5 mg calcium pantothenate, 4 mg niacin, 100 mg choline chloride,

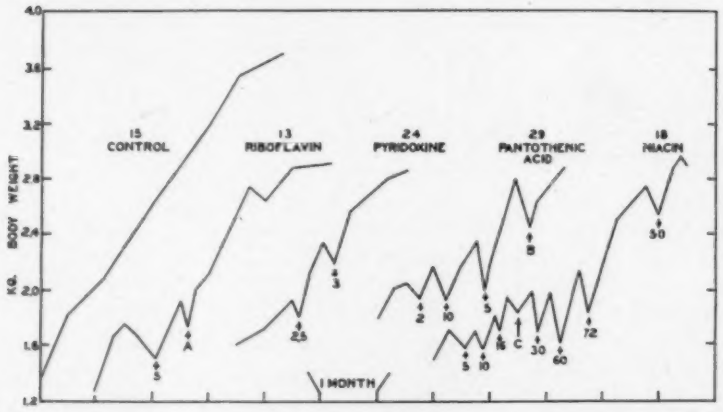

Fig. 1 Growth of fox pups demonstrating the effect of omitting 1 vitamin from the basal ration. Numbers (i.e., 5) equal mg of therapy of the specific vitamin omitted. Fox 15 — complete basal. No. 13 — basal ration minus riboflavin. A, started 0.125 mg per 100 gm ration. No. 24 — basal ration minus pyridoxine. No. 29 — basal ration minus pantothenic acid. B, 20 mg calcium pantothenate orally plus 5 mg intravenously in a solution of glucose and saline. No. 18 — started on the basal ration minus niacin and folic acid. C, 30 mg niacin orally and started 0.1 mg folic acid per 100 gm of ration.

0.1 mg folic acid, 0.025 mg biotin, 25 mg i-inositol, 50 mg p-aminobenzoic acid, 2 mg alpha-tocopherol and 0.5 mg 2-methyl-1,4-naphthoquinone. Growth curves of a typical fox pup from each series of experiments are given in figure 1. Table 1 summarizes the blood findings.

Riboflavin

Red fox pups nos. 13, 14 and silver pup 19 received the basal ration minus riboflavin. After 2 weeks on experiment the rate

of growth of all the pups showed a definite decrease. Pup 19 was fed a single supplement of 2 mg riboflavin and normal growth resumed. However, 3 weeks later failure was extremely sudden, and the animal died before another supplement could be given. After 3 to 4 weeks on the riboflavin deficient ration, pups 14 and 13 exhibited acute deficiency

TABLE 1

Ration = Basal — Pyridoxine.

PUP. NO.	DAYS ON EXP'T.	WEIGHT	HEMOGLOBIN	HEMATOCRIT	R.B.C.
		kg	*gm %*	*%*	*per cm³*
24	1	1.60	8.99	26.9	....
	31	1.86	9.26	22.0	7.17
	49	2.22	6.86	17.0	4.35
	49	Fed 3 mg pyridoxine orally and started 0.2 mg pyridoxine per 100 gm ration			
	63	2.62	10.90	34.0	9.0
25	1	1.55	7.35	30.8	
	31	1.78	8.21	17.5	5.67
	49	2.09	5.60	12.5	4.22
	52	died			
28	1	2.96	14.16	42.0	10.11
	69	3.11	14.67	43.8	10.33
	101	3.20	10.89	37.0	10.22
		Started 0.20 mg pyridoxine per 100 gm ration			
	121	3.33	15.48	54.2	10.94
Av. of	49	2.89	11.80	35	8.70
4 pups on	70	3.56	12.49	40.0	9.24
complete	98	4.07	14.63	42.4	9.60
basal					

symptoms characterized by muscular weakness followed by clonic spasms and coma. Pup 13 was fed 1 dose of 5 mg riboflavin orally and pup 14 received 5 mg orally and 1.25 mg intravenously. Within 2 hours the animals regained muscular control and consciousness. A normal gain in body weight followed. Three weeks later growth in both animals was retarded, opacity of the lens and diminution of pigment production in the underfur and guard hair was noted. Therapy

consisting of 0.125 mg and 0.250 mg riboflavin per 100 gm of ration for pups 13 and 14, respectively, was initiated when the first signs of muscular weakness were noted. Both animals gained weight rapidly; however, after a period of 9 weeks following the daily supplementation of the ration with ribo-

Fig. 2 Pelts of wild red fox pups 13 and 14 which developed a riboflavin deficiency and then received 0.125 mg and 0.250 mg riboflavin, respectively, per 100 gm of ration. Pelt on the right is that of a red fox pup receiving the complete basal ration supplemented with liver. (Note the white underfur on pelts 13 and 14).

flavin, pup 14, which received 0.25 mg, weighed 740 gm more than pup 13.

The lack of proper pigmentation of the fur continued, and 6 to 7 weeks after riboflavin therapy was initiated the underfur and guard hair were white. Pigmentation of the guard hair gradually returned and was approaching normal, whereas the underfur was still white when the pups were pelted (fig. 2). The symptoms, such as loss in weight, muscular weakness, coma and opacity of the lens were similar to those observed in riboflavin deficient dogs (Potter et al., '42).

Pantothenic acid

Five pups, 4 red and 1 silver, were placed on the basal ration minus calcium pantothenate. After 2 to 3 weeks on experiment growth of the pups ceased although food consumption remained normal. Failure was extremely sudden in pups 11 and 12 and death resulted when the animals had been on experiment for 26 and 27 days, respectively. When pup 30 had been on experiment for 18 days and a definite loss in body weight was evident, the ration was supplemented with 0.25 mg calcium pantothenate per 100 gm ration. The gain in body weight was rapid for 8 weeks; however, failure was sudden and the animal died before additional therapy could be given.

Pup 16 responded once to calcium pantothenate when in a mild state of deficiency. Later the more severe form was allowed to develop, which was characterized by a deep coma. Intravenous injection of a solution of calcium pantothenate, glucose and saline resulted in relief from the coma in 1 hour; however, the pup was found dead the following morning. Pup 29, as shown in figure 1, after responding 3 times to calcium pantothenate therapy was allowed to develop a severe deficiency which was characterized by clonic spasms and this condition was followed by coma. A solution of calcium pantothenate, glucose and saline was injected intravenously, and 30 minutes after therapy the pup had recovered from the coma and was running about the cage. A significant increase in body weight followed.

Necropsies performed on all pups immediately after death revealed fatty degeneration of the liver, catarrhal gastroenteritis, and cloudy swelling and congestion of the kidneys. The deficiency symptoms, the dramatic response to pantothenic acid, and the results of macroscopic pathological examination of pups which died of the deficiency were similar to those observed in dogs (Schaefer et al., '42a). The preliminary results obtained with pup 30 indicates that the requirement for pantothenic acid is greater than 0.25 mg per 100 gm ration.

Niacin

Silver pups nos. 17, 18 and 23 received the basal ration minus niacin and folic acid. Within 2 weeks anorexia was apparent and the pups failed to gain weight. The administration of 5 to 30 mg niacin resulted in resumption of growth. During the first 5 weeks on experiment pup 18 (fig. 1) developed 3 deficiencies with subsequent responses to niacin. The ration was then supplemented with 0.1 mg folic acid per 100 gm ration and 4 more deficiencies, with subsequent response to single dosages of varying levels of niacin, were obtained. After 3 deficiency and treatment periods, during the first 6 weeks on experiment, the ration for pup 17 was supplemented with folic acid. One week later loss in body weight was evident and the oral administration of 26 mg niacin failed to alleviate the deficiency. The subsequent feeding of 70 gm fresh liver produced a rapid gain in body weight.

Pup 23 during the first 4 weeks on experiment developed 2 deficiencies with subsequent responses to niacin. The ration was then supplemented with folic acid. After 2 additional deficiencies and responses to niacin the animal lost weight rapidly. The oral administration of 86 mg niacin, 2 mg folic acid, 10 times the daily supplement of the remainder of the B-complex and the intramuscular injection of 20 mg nicotinamide, failed to correct the deficiency and the animal died. The failure of the animals to respond to niacin after repeated deficiencies, and the rapid response to fresh liver is very similar to the results obtained with dogs given the same treatment (Ruegamer et al., '47).

Typical blacktongue symptoms noted in dogs (Schaefer et al., '42b) such as loss in weight, anorexia, inflammation of the gums, palatine redness, and diarrhea were noted in the foxes where a severe deficiency was allowed to develop. The requirement for nicotinic acid as calculated by single dose feedings for growing pups varied greatly among assays with the same pup and among pups (range of 0.39 to 2.0 mg per kg body wt. per day).

Pyridoxine

Silver pups 24 and 25 and red pup 28 were placed on the basal ration minus pyridoxine. Five ml blood samples were collected from the radial vein at selected intervals. Hemoglobin determinations were done according to the method of Evelyn ('36); hematocrit readings were obtained with Wintrobe tubes. Blood analyses are given in table 1. Anorexia and arrest in growth occurred in pups 24 and 25 after being on experiment for 5 weeks. 2.5 mg pyridoxine were administered orally and the animals gained weight for 3 weeks. At this time the animals again developed anorexia, appeared listless and were anemic. Hemoglobin values were 6.86 and 5.6 gm % for pups 24 and 25, respectively, as compared to 11.8 gm % for animals receiving the complete basal ration. Pup 25 was in a comatose state when pyridoxine therapy was started and failed to recover. The oral feeding of 3 mg pyridoxine and supplementation of the ration thereafter with 0.2 mg per 100 gm to pup 24 resulted in a rapid response in growth and alleviation of the anemia. The animal was killed accidentally before it could be determined whether complete remission of the anemia would result from continued pyridoxine therapy.

Pup 28 was approximately 20 weeks of age when placed on experiment. The body weight curve showed a plateau and after 17 weeks on experiment the hemoglobin level dropped from 14.67 to 10.89 gm %. At this time the ration was supplemented with 0.20 mg pyridoxine per 100 gm ration. Hemoglobin was increased to 15.48 gm %; however, no significant increase in body weight was noted.

DISCUSSION

From these experiments it is evident that fox pups require riboflavin, pantothenic acid, niacin and pyridoxine. The deficiency symptoms observed when 1 of the above vitamins is omitted from the ration closely resemble those reported in similar studies with dogs (Potter et al., '42; Schaefer et al., '42a, b; McKibbin et al., '42). The rapidity with which the

deficiencies develop, especially of riboflavin, pantothenic acid or niacin, is very striking and emphasizes the importance of insuring adequate quantities of these vitamins in rations for growing fox pups.

Although our experiments were not designed to study the exact requirements of fox pups for the above vitamins, the following minimum and maximum levels may be suggested for future work: Riboflavin — greater than 0.125 mg and less than 0.4 mg per 100 gm of ration (0.2 mg for the dog). Pantothenic acid — greater than 0.25 mg and less than 1.5 mg per 100 gm of ration (0.20 mg for the dog). Niacin — 0.39 mg to 2.0 mg per kg of body weight per day (0.25 mg to 0.36 mg for the dog) as calculated from single dose feeding. Pyridoxine — less than 0.20 mg per 100 gm of ration (0.12 mg for the dog).

The changes occurring in the pigmentation of the fur of the foxes on the riboflavin experiment are of extreme interest. This diminution of red color may have been due to riboflavin and in part or entirely to the lack of unknown factors supplied by liver, since a depigmentation of the underfur has been observed in silver fox pups receiving the complete basal ration (Schaefer et al., '47). The quality of the fur was not affected.

SUMMARY

The importance of riboflavin, pantothenic acid, niacin and pyridoxine in the rations for growing fox pups has been clearly established. The deficiency symptoms when 1 of the above vitamins is missing from the ration closely resemble those observed in dogs. The rate with which the deficiencies develop is as rapid, and in numerous instances even more rapid, in fox pups than in dogs.

ACKNOWLEDGMENTS

We wish to acknowledge our indebtedness to Merck and Company, Rahway, N. J., for the crystalline vitamins; and to Lederle Laboratories, Inc., Pearl River, N. Y., for the synthetic folic acid.

LITERATURE CITED

EVELYN, K. A. 1936 Photoelectric colorimeter. J. Biol. Chem., *115:* 63.

MCKIBBIN, J. M., A. E. SCHAEFER, D. V. FROST AND C. A. ELVEHJEM 1942 Studies on anemia in dogs due to pyridoxine deficiency. J. Biol. Chem., *142:* 77.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.

POTTER, R. L., A. E. AXELROD AND C. A. ELVEHJEM 1942 The riboflavin requirement of the dog. J. Nutrition, *24:* 449.

RUEGAMER, W. R., NANCY TORBET AND C. A. ELVEHJEM 1947 Hematological and growth responses in dogs given liver extracts containing the antipernicious anemia factor. Fed. Proc., *6:* 287.

SCHAEFER, A. E., J. M. MCKIBBIN AND C. A. ELVEHJEM 1942a Pantothenic acid deficiency studies in dogs. J. Biol. Chem., *143:* 321.

———— 1942b Nicotinic acid deficiency studies in dogs. J. Biol. Chem., *144:* 679.

SCHAEFER, A. E., C. K. WHITEHAIR AND C. A. ELVEHJEM 1947 Purified rations and the requirement of folic acid for foxes. Arch. Biochem., *12:* 349.

DIET OF MOTHER AND BRAIN HEMORRHAGES IN INFANT RATS[1]

E. E. BROWN, J. F. FUDGE AND L. R. RICHARDSON
Division of Chemistry, Texas Agricultural Experiment Station, Agricultural and Mechanical College of Texas, College Station

TWO FIGURES

(Received for publication May 5, 1947)

Several investigators have shown that young rats grow to maturity at a rapid rate and bear litters when fed a synthetic ration containing vitamins A and D, alpha tocopherol, thiamine, riboflavin, pyridoxine, pantothenic acid, niacin and choline. The diets in some cases (Richardson, Long and Hogan, '42; Richardson and Hogan, '46; Climenko and McChesney, '42; Ershoff, '46) contained 2-methyl-1,4-naphthoquinone. In other cases (Jukes, '40; Unna, '40; Henderson et al., '42; Vinson and Cerecedo, '44) this compound was not added to the diet but the vitamin probably was supplied by some constituent such as corn oil or cottonseed oil, or by bacterial synthesis in the digestive tract of the mother.

Synthesis of vitamin K in the intestinal tract of the rat has been demonstrated by several investigators. Greaves ('39) has reported a decrease in blood prothrombin and a prolonged bleeding time in rats with bile fistulas. Oral administration of large amounts of vitamin K concentrates or smaller amounts combined with bile salts restored clotting time to normal. Black et al. ('42), Day et al. ('43), and Kornberg et al. ('44) observed a high incidence of severe hypoprothrombinemia in

[1] Authorized for publication as Technical Article no. 1039 of the Texas Agricultural Experiment Station.

rats receiving diets that contained sulfonamides to prevent sufficient intestinal synthesis of vitamin K. When the vitamin K was fed with sulfonamide the hypoprothrombinemia did not develop. These data indicate that vitamin K acts in rats as it does in other animals to maintain a normal prothrombin level in the blood. The Syrian hamster requires (Hamilton and Hogan, '44) vitamin K for a normal rate of growth and presumably for normal reproduction. Moore et al. ('42) found a low prothrombin level in the blood of pregnant rabbits that had received diets deficient in vitamin K. These females aborted at the end of the first trimester of gestation.

Some investigations have been carried out in this laboratory during the past year on the relation of the diet of the mother to abnormalities in the offspring. A high incidence of brain hemorrhage has occurred in the offspring of mothers which received a synthetic diet deficient in vitamin K and low in fat. Some of the observations on this abnormality are described in this report.

EXPERIMENTAL

The composition of the basal diet of the mothers is given in table 1. This diet contains all the recognized vitamins with the exception of vitamin C and pteroylglutamic acid, and it has been reasonably satisfactory in reproduction studies over

TABLE 1

Composition of basal diet.

	gm	*Vitamins per 100 gm*	
Casein	25	Vitamin A	3000 I.U.
Cerelose	57	Vitamin D	425 I.U.
Woodpulp	3	Alpha tocopherol [1]	2.5 mg
Salt mixture [2]	5	Menadione [1]	2.5 mg
Lard	10	Thiamine [1]	1.0 mg
Choline [1]	0.1	Riboflavin [1]	1.0 mg
Inositol [1]	0.01	Pyridoxine [1]	1.0 mg
p-Aminobenzoic acid [1]	0.05	Calcium pantothenate [1]	4.0 mg
		Niacin [1]	5.0 mg
		Biotin [1]	0.02 mg

[1] Supplied by Merck and Co., Rahway, N. J.

[2] Richardson and Hogan ('46).

a period of several years. Vitamins A and D were supplied by percomorph oil.[2] Lard and vitamin K were the only variable constituents of the experimental diets. The amounts of these constituents in the different diets and the observations on the young of the females which received the diets are summarized in table 2. The females in group 1 received the basal diet, diet A, which contains both lard and vitamin K. Those in group 2 received diet B which contains vitamin K but no lard. Those in group 3 received diet C which contains lard but no vitamin K, and those in group 4 received diet D which contains neither lard nor vitamin K.

TABLE 2

Relation of the diet of the mother to brain hemorrhages in infant rats.

GROUP NO.	DIET NO.	VARIABLE CONSTITUENTS		YOUNG				
		Lard	Vitamin K[1]	Born	Hemorrhagic brains		Weaned	
		%	*mg/100 gm*	*No.*	*No.*	%	*No.*	%
1	A	10	2.5	167	0	0	89	53.2
2	B	..	2.5	142	0	0	49	34.5
3	C	10	..	63	0	0	31	49.2
4	D	..	..	157	82	52.2	18	11.4

[1] 2-Methyl-1,4-Naphthoquinone.

Female rats were given either diet A or diet C at 28 days of age and continued on the same diet until they weighed 175 to 200 gm. At this time they were either continued on the same diet or transferred to another experimental diet and mated with a male from the stock colony. The females which were transferred to diet B had been reared on diet A and those which were transferred to diet D had been reared on diet C.

Diet of mother and brain hemorrhages. When the diet contained both lard and vitamin K (group 1) the offspring were normal and 89 out of 167 (53.2%) were weaned. Likewise the offspring were normal when the diet of the mother contained either vitamin K (group 2) or lard (group 3) separately,

[2] Mead, Johnson and Company.

but a higher percentage of the young was weaned when the diet contained lard without vitamin K than was weaned when it contained vitamin K without lard. There was a high incidence of brain hemorrhages in the offspring of females (group 4) which received neither lard nor vitamin K. Brain hemorrhages occurred in 82 out of 157 (52.2%) of the young and only 18 (11.4%) were weaned.

Incidence of brain hemorrhages. The observations on the young which are given in group 4 of table 2 have been rearranged in table 3 to show the incidence of brain hemorrhages in the first litters as compared to that in subsequent litters.

TABLE 3

Incidence of brain hemorrhages in first as compared to subsequent litters.

GROUP NO.	DIET NO.	NO. OF FEMALES	LITTER SEQUENCE	BORN	YOUNG HEMORRHAGIC BRAINS		WEANED	
				No.	*No.*	*%*	*No.*	*%*
4a	D	6	1	38	17	44.7	4	10.5
4b	D	12	1	58	20	34.5	14	24.1
			2 to 6	61	45	73.7	0	0
4ac[1]	C	6	2 to 4	29	0	0	17	58.6

[1] Same females as described in 4a. After a female had borne 1 litter, she was transferred to diet C which contained lard but no vitamin K.

The 6 females in group 4a were each allowed to produce 1 litter on diet D. A total of 38 young were born in the first litters and 17 (44.7%) developed severe brain hemorrhages. Only 4 (10.5%) were weaned. The females (group 4ac) were then transferred to diet C and allowed to bear additional litters until it was evident that no more young would be born. A total of 29 young were born in subsequent litters and none were hemorrhagic. Seventeen out of the 29 (58.6%) were weaned. The addition of lard to the diet decreased the incidence of brain hemorrhages to zero and increased the per cent of young weaned.

The 12 females in group 4b received diet D from the time they were mated until the observations were discontinued. A

total of 58 young were born in the first litters. Severe hemorrhages occurred in 20 (34.5%), and 14 young (24.1%) were weaned. The same females produced 61 young in subsequent litters and brain hemorrhages occurred in 45 (73.7%) and none were weaned. Although brain hemorrhages constituted the most characteristic abnormality, hemorrhages occurred also in the body cavity in a few young.

Whole blood coagulation time. The coagulation time of blood of 12 mothers which produced hemorrhagic young and of 6 young with visible hemorrhages in the brain was determined by Duke's capillary tube method. It ranged from 60 to 120 seconds in both the mothers and the young and is considered normal since it did not differ from that of rats in the stock colony.

Since both lard and vitamin K in the diet of the mother protect the young from hemorrhages, it seemed desirable to test the vitamin K activity of lard with chicks. Day old New Hampshire Red chicks were used in these tests and the experimental period was 2 weeks. The basal diet was similar to that described by Richardson, Hogan and Karrasch ('42). Pteroylglutamic acid was supplied by 10% of ether-extracted dried brewers' yeast. The average coagulation times of blood of chicks which received diets containing different amounts of lard with and without vitamin K for 2 weeks are summarized in table 4. When the diet contained vitamin K (groups

TABLE 4

Average coagulation time of blood of chicks receiving diets that contain different amounts of lard with and without vitamin K.

GROUP NO.	LARD	VITAMIN K	COAGULATION TIME [1]	
			No. of chicks	Min.
	%	*mg/100 gm*		
1 C	10	2.5	5	3.3
2 C	0	2.5	5	3.8
3 C	0	0	4	30 plus
4 C	5	0	4	30 plus
5 C	10	0	4	22
6 C	20	0	4	24

[1] Experimental period — 2 weeks.

1C and 2C) the whole blood coagulation time was normal regardless of whether or not the diet contained lard. When the diet did not contain vitamin K (groups 3C, 4C, 5C and 6C) the whole blood coagulation time was prolonged regardless of the amount of lard present. However, it was longer in the groups which received no lard (group 3C) and only 5% of lard (group 4C) than in groups 5C and 6C which received 10 and 20% of lard, respectively. These tests show that lard, when fed to chicks, supplies a small but insignificant amount of vitamin K. This small amount might be sufficient when fed to rats at a level of 10% to meet the requirement for reproduction under normal conditions. However, we do not believe that this explanation is entirely adequate, in view of the fact that the whole blood coagulation time of hemorrhagic rats is normal. If the reports by Black et al. ('42), Day et al. ('43), and Kornberg et al. ('44) that a prolonged coagulation time and a hypoprothrombinemia are typical of a vitamin K deficiency are correct, these data suggest that brain hemorrhages are not due entirely to a deficiency of this vitamin. A possible explanation of this discrepancy would be that some substance is produced in the body under normal conditions which maintains normal capillary fragility. This substance is not synthesized when the diet is low in fat and deficient in vitamin K at the same time.

Age at death. The age at which the infant rats died with brain hemorrhage is shown in table 5. Most of the young which died with brain hemorrhages were either born dead or died within 24 hours after birth. The hemorrhages were

TABLE 5

Age at death of rats with hemorrhagic brains.

Age in days	*No. of rats*
Birth	22
1	45
2 to 5	3
8 to 11	5
15 to 18	4
20 to 21	3

visible in a few young while they were still living, but death always occurred within a few hours when the hemorrhages became visible in living young that were less than 5 days old. The larger rats usually became unstable 2 to 3 days before death and in a few cases a dark area was visible underneath the skull. Every rat which developed these symptoms was autopsied at death and brain hemorrhages had occurred in every case. Hemorrhage occurred after the fifth day only in first litters.

In a second test brain hemorrhages developed in only 10 out of 72 young which were born to 8 females. The incidence of brain hemorrhages was about the same in the first as it was in the second litters. So far we are unable to account for the low incidence of hemorrhages in this series.

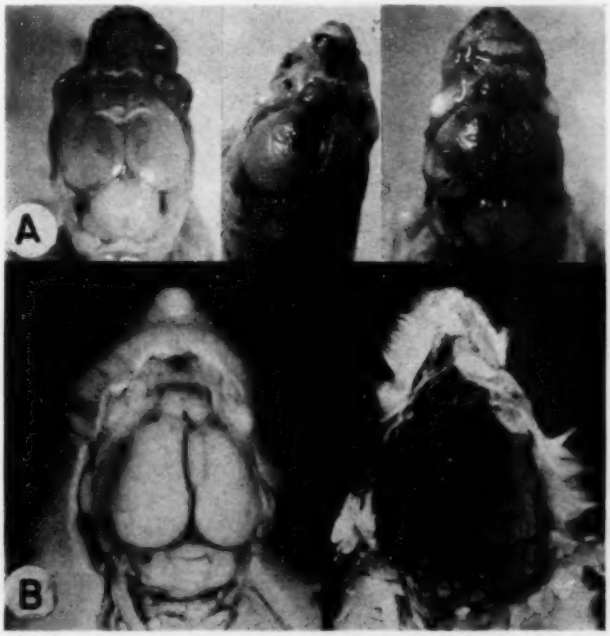

Fig. 1 Normal and hemorrhagic brains. (A) Day-old rats. (B) Twenty-one-day-old rats.

Photographs of the brains of a normal and of hemorrhagic rats 1 day and 21 days old are shown in figure 1. Microphotographs [3] of sections of a normal and of a hemorrhagic brain are shown in figure 2. Large areas of blood in the tissues are visible in the hemorrhagic brain. In the preparation of the slides for the microphotographs the tissue was fixed and

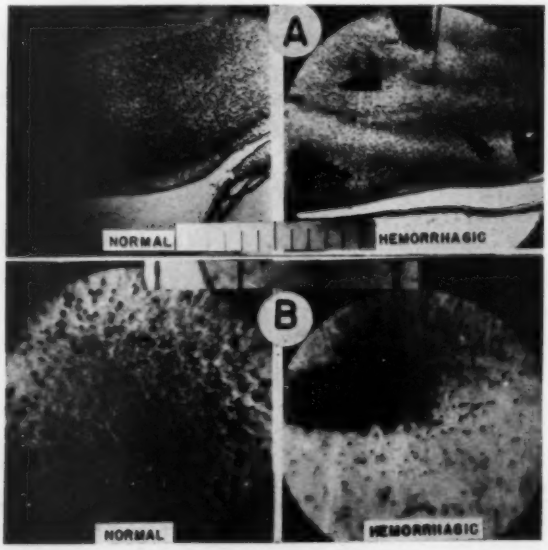

Fig. 2 Sagittal section of brain through cerebrum of a normal and of a hemorrhagic rat. A, low power; B, high power. One division of stage micrometer scale equal to 0.1 mm.

decalcified in Bouin's fixative and stained by Lillie's modification of Masson's trichrome stain.

Reports of brain hemorrhages by other workers. Brain hemorrhages in infant rats have been reported previously by 2 groups of workers. Moore et al. ('27) reported brain hemorrhages in a few of the young born to females which

[3] We are indebted to Dr. Sidney O. Brown, Associate Professor of Biology, for the preparation of sections and slides and for the microphotographs.

had been reared and allowed to bear young on a synthetic diet that contained 2% of dried yeast as the only source of the water-soluble vitamins. The diet contained 3% of hydrogenated cottonseed oil [4] as a source of fat. The hemorrhages were thought to be due to a low level of vitamin B because they did not occur when the yeast was increased to 7%. Jervis ('42) reported the occurrence of brain hemorrhages in infant rats from mothers who were fed a synthetic diet deficient in choline. Three females received the experimental diet which contained 10% hydrogenated cottonseed oil and each female received a daily supplement of 100 μg each of thiamine, pyridoxine, riboflavin and calcium pantothenate and 6 mg of niacin. The 2 control females received essentially the same diet with 100 mg of choline added to each 100 gm of the diet. The young from the mothers on the choline deficient diet ceased to grow at the tenth to twelfth day and 50% of them died with severe nervous symptoms. Extensive hemorrhagic lesions of the cerebellum were the most characteristic abnormality found on histological examination of the nervous system of 5 young which exhibited the very severe nervous symptoms. Vitamin K was not included in the diet of the females described in either of these reports and both investigations used hydrogenated cottonseed oil [4] as the source of fat. We are investigating the effect of this particular fat on the production of brain hemorrhages at the present time, but it seems very probable that the hemorrhages described in the previous reports, as well as those described here, are due to the same deficiency.

SUMMARY

Female rats were reared to maturity on a synthetic diet that was deficient in vitamin K. If lard was removed from their diet and the females allowed to bear litters, a high incidence of brain hemorrhages occurred in the offspring. The hemorrhages did not occur when the diet contained either lard or vitamin K.

[4] Crisco.

The incidence of hemorrhage in first litters was 34.5 to 44.17% and in subsequent litters, 73.7%.

Most of the young that died with brain hemorrhages were either born dead or died within 24 hours after birth. A few young in the first litters developed hemorrhages between the eleventh and twenty-first days.

The whole blood coagulation time of females which produced hemorrhagic young and of young with visible hemorrhages was normal. This shows that the prothrombin level was not abnormally low and it is suggested that some substance which acts normally to maintain capillary strength is not synthesized by the body when the diet is low in fat and deficient in vitamin K. An assay with chicks shows that lard may contain a small but insignificant amount of vitamin K.

LITERATURE CITED

BLACK, S., R. S. OVERMAN, C. A. ELVEHJEM AND K. P. LINK 1942 The effect of sulfaguanidine on rat growth and plasma prothrombin. J. Biol. Chem., *145:* 137.

CLIMENKO, D. R., AND E. W. MCCHESNEY 1942 Role of inositol and p-aminobenzoic acid in normal lactation. Proc. Soc. Exp. Biol. Med., *51:* 157.

DAY, H. G., K. G. WAKIM, M. M. KRIDER AND E. E. O'BANION 1943 Effects of cecectomy, succinylsulfathiazole and p-aminobenzoic acid on vitamin K synthesis in the intestinal tract of rats. J. Nutrition, *26:* 585.

ERSHOFF, B. H. 1946 Effects of massive doses of p-aminobenzoic acid and inositol on reproduction in the rat. Proc. Soc. Exp. Biol. Med., *63:* 479.

GREAVES, J. D. 1939 The nature of the factor which is concerned in the loss of blood coagulability of bile fistula and jaundiced rats. Am. J. Physiol., *125:* 423.

HAMILTON, J. W., AND A. G. HOGAN 1944 Nutritional requirements of the Syrian hamster. J. Nutrition, *27:* 213.

HENDERSON, L. M., J. M. MCINTIRE, H. A. WAISMAN AND C. A. ELVEHJEM 1942 Pantothenic acid in the nutrition of the rat. J. Nutrition, *23:* 47.

JERVIS, G. A. 1942 Occurrence of brain hemorrhages in choline deficient rats. Proc. Soc. Exp. Biol. Med., *51:* 193.

JUKES, T. H. 1940 Reproduction in rats on synthetic B-complex supplement. Proc. Soc. Exp. Biol. Med., *45:* 625.

KORNBERG, A., F. S. DAFT AND W. H. SEBRELL 1944 Production of vitamin K deficiency in rats by various sulfonamides. U. S. Pub. Health Repts., 59: 832.

MOORE, C. U., J. L. BRODIE AND R. B. HOPE 1927 Some effects upon the young of inadequate maternal diets. I. Polyneuritis and hemorrhages. Am. J. Physiol., *82:* 350.

MOORE, R. A., I. BITTINGER, M. L. MILLER AND L. M. HELLMAN 1942 Abortion in rabbits fed a vitamin K-deficient diet. Am. J. Obstet. Gynecol., *43:* 1007.

RICHARDSON, L. R., AND A. G. HOGAN 1946 Diet of mother and hydrocephalus in infant rats. J. Nutrition, *32:* 459.

RICHARDSON, L. R., A. G. HOGAN AND R. J. KARRASCH 1942 A liver concentrate as a source of unrecognized vitamins required by the chick. J. Nutrition, *24:* 65.

RICHARDSON, L. R., BARBARA LONG AND A. G. HOGAN 1942 The vitamins required for growth and reproduction in the rat. Abst. of Papers, 103rd meeting, Am. Chem. Soc., Memphis.

UNNA, K. 1940 Effect of pantothenic acid on growth and reproduction of rats on synthetic diets. Am. J. Med. Sci., *200:* 848.

VINSON, L. J., AND L. R. CERECEDO 1944 Growth, reproduction and lactation in rats maintained through 4 generations on highly purified diets. Arch. Biochem., *3:* 389.

AVAILABILITY OF CAROTENE FROM CARROTS AND FURTHER OBSERVATIONS ON HUMAN REQUIREMENTS FOR VITAMIN A AND CAROTENE [1]

ELIZABETH CROFTS CALLISON AND ELSA ORENT-KEILES
Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, U. S. Department of Agriculture, Washington, D. C.

(Received for publication April 17, 1947)

Carrots occupy a unique place among the vegetables as a potential contributor to the vitamin A value of the *human* diet in the United States not only because they have by far the highest carotene content of any food commonly eaten by man (U. S. Bureau of Human Nutrition and Home Economics, '45), but also because they are consumed in large quantities (U. S. Bureau of Human Nutrition and Home Economics and National Research Council, '44). However, there is evidence that the ability of the human body to absorb and utilize the large amounts of carotene present in carrots is very limited (Eekelen and Pannevis, '38; Kreula and Virtanen, '39; Lister Institute and Medical Research Council, Vitamin A Sub-committee of the Accessory Food Factors Committee, '45).

Employing the dark adaptation reaction as a criterion of the vitamin A status of the individual, Booher and Callison ('39) and Booher, Callison and Hewston ('39) in this laboratory have established a procedure for measuring the degree of utilization of vitamin A by man and have determined the

[1] This research was supported by an allotment made by the Secretary of Agriculture from Special Research Funds (Bankhead-Jones Act of June 29, 1935).

human requirements for vitamin A when the sources were cod liver oil, crystalline carotene in cottonseed oil, frozen spinach or frozen peas. This report presents the results of an investigation on the utilization of carotene from carrots by the human body.

EXPERIMENTAL

The same plan of experiment and basal vitamin A-deficient diet described in detail in the previous papers (Booher and Callison, '39; Booher, Callison and Hewston, '39) were used in these experiments. Four young adults, whose physical characteristics are summarized in table 1, subsisted for

TABLE 1

Physical characteristics of subjects.

SUBJECT NUMBER, SEX, AGE IN YEARS	HEIGHT IN CM	WEIGHT IN KG	TIME REQUIRED FOR DEPLETION (PERIOD 1) IN DAYS	VISUAL THRESHOLDS EXPRESSED IN LOG $\mu\mu$ LAMBERT					
				Before depletion Dark adapted		After depletion Dark adapted		After recovery with carrots Dark adapted	
				6 min.	30 min.	6 min.	30 min.	6 min.	30 min.
6-M-18	172.7	57.9	12 + 92 [1]	5.76	2.97	6.22	3.64	5.95	3.05
7-M-20	179.1	80.0	176	5.73	3.17	5.72	3.73	5.72	3.21
8-M-19	167.6	57.5	154	5.64	2.95	6.20	3.65	5.75	3.05
9-F -21	160.0	52.1	60	5.96	3.08	6.20	3.47	5.60	3.10

[1] Depletion period interrupted for approximately 4 weeks by appendectomy.

periods of from 7 months to 1 year on the basal diet deficient in vitamin A value but adequate in calories, minerals and all other known nutrients. The daily intake of vitamin A from this diet averaged from 70 to 80 I.U. and consisted entirely of vegetable carotenoids present in small amounts in some of the fruits and vegetables.

Dark adaptation tests were made at frequent intervals by means of the Hecht-Shlaer visual adaptometer. When the visual threshold after 30 minutes dark adaptation following a standardized exposure to light had increased to a value of 0.5 to 0.7 log unit above the subject's normal average threshold, the daily inclusion in the diet of carrots in weighed

amounts was begun. An amount of carrots calculated, on the basis of previous experience, to be not quite adequate for the restoration of normal vision was given. When no further improvement in dark adaptation from this subminimal dose was apparent, the size of the carrot supplement was changed by a small increment. This procedure was repeated until a daily intake of carrots was reached which sufficed to restore dark adaptation to normal and maintain it so over a 2-week period.

In order, also, to determine the effect of the degree of cooking upon the availability of the vegetable carotene to the organism, the vitamin A requirement of each subject was determined twice, once when the carrots were fed quick-frozen with no preparation other than thawing, and again after the thawed carrots had been cooked in a pressure saucepan by a standard method. The subjects were depleted of their vitamin A reserves a second time, prior to feeding the cooked carrots.

One lot of market carrots was quick-frozen for this purpose according to the method described by Tressler and Evers ('43) and stored at a temperature of — 40°C. Portions were removed daily as required by the experimental subjects during the supplementation periods. From each portion so removed, an aliquot was reserved for parallel chemical and biological determinations of carotene content and vitamin A value. The daily aliquots of the cooked and uncooked carrots for each of the 4 subjects were pooled separately for the carotene assay.

Biological assay

Because of the structure of carrots, the problem of obtaining truly representative small samples, such as are used in bioassay, is unusually difficult. The complexities are increased when a chemical analysis of a similar sample is desired. The procedure finally adopted in this study consisted of cutting from a carrot slice a wedge-shaped piece to be fed to the experimental animal. Thus the same propor-

tion of phloem to cortex was present in the piece as in the whole slice. The remainder of the slice was preserved for chemical analysis by storing at — 40°C. The bioassays were conducted essentially according to the procedure outlined in the U. S. Pharmacopoeia (Twelfth ed.), 108 animals being used. Crystalline β-carotene[2] in cottonseed oil was employed as the standard, since it has been found that the U.S.P. reference cod liver oil no. 2 is unsuitable for use as a standard of reference (Callison and Orent-Keiles,' 45).

Chemical analysis

The pooled aliquots of carrots, 1 cooked and 1 uncooked for each subject, were extracted according to the method of Moore and Ely ('41). The extracts were chromatographed on a 1:2 mixture of magnesium oxide [3] and Hyflo Supercel. The α- and β-carotene fractions were eluted separately with purified petroleum ether, fraction F, and absorption of each fraction was determined at 450 mμ, using a Coleman spectrophotometer, Model 10-S. The amount of α- or β-carotene present in its respective fraction was calculated from a standard β-carotene curve obtained on the same instrument. The standard β-carotene curve was used in estimating both α- and β-carotene, since Zscheile, White, Beadle and Roach ('42) have shown that the absorption coefficients at 450 mμ are practically identical for these 2 isomers. The bands at the top of the magnesia column, which amounted to only 4% of the total carotenoids, were not investigated further.

RESULTS AND DISCUSSION

The time required for the first depletion of these subjects ranged from 2 to 6 months, reflecting the great individual variation in vitamin A reserves that had been observed in previous experiments in this laboratory. The second deple-

[2] S.M.A. β-carotene.

[3] E. S. Good reagent, tested in this laboratory for ability to give good separation and recovery of α- and β-carotene.

tion period was much shorter and more uniform. In every case the scotopic visual threshold began to rise as soon as the carrot supplement was discontinued and within 13 to 16 days had reached a level 0.5 to 0.7 log units above the subject's normal average threshold. At this time feeding of the second carrot supplement was begun. Both periods of depletion were in progress during the spring and summer months, so that the seasonal elevation of visual threshold associated with winter, which has been reported by English investigators (Lister Institute and Medical Research Council, Vitamin A Sub-committee of the Accessory Food Factors Committee, '45) was not encountered.

TABLE 2

Vitamin A potency of carotene-containing supplements as measured by chemical and bioassay methods.

SUPPLEMENT	VITAMIN A VALUE MEASURED BY		DIFFERENCE BETWEEN PREDICTED AND BIOASSAY VALUES
	Chemical Analysis	Bioassay	
	I.U./gm	*I.U./gm*	%
Crystalline carotene in cottonseed oil [1]	1.44×10^6	1.67×10^6	+ 15.9
Carrots, frozen, cooked [2]	146	50.5	— 65.4
Peas, frozen, cooked [1]	6.2	7.0	+ 12.9
Spinach, frozen, cooked [1]	85	97.5	+ 14.7

[1] U.S.P. reference cod liver oil no. 1 used as standard in bioassay.
[2] S.M.A. β-carotene used as standard in bioassay.

Cooking, over and above the amount required for blanching the carrots preparatory to freezing, caused no differences in either the carotene content of the vegetable or the ability of the human to utilize the carotene. The same amount of carrots whether cooked or uncooked was required by each subject for restoration of normal vision.

There was a marked discrepancy between the vitamin A value of the carrots calculated from the chemical analysis, using 0.6 μg β-carotene and 1.2 μg α-carotene, respectively, as equivalent to 1 I.U. of vitamin A, and that same value measured directly by the rat-growth bioassay procedure (table 2).

Chemical analyses of the samples were in close agreement and gave an average of 77.8 μg β-carotene and 19.4 μg α-carotene or a calculated total of 146 I.U. vitamin A value per gm of carrots. The vitamin A activity of duplicate samples of carrots determined by rat-growth bioassay was 50.5 I.U. per gm, equivalent to only one-third of the provitamin A shown to be present in the vegetable by chemical analysis.

It is obvious (table 3) that there can be a three-fold difference in the statement of the vitamin A requirement of these human subjects, depending upon which value, chemical or biological, is used in calculating the potency of the carrots from which practically the entire vitamin A intake of the

TABLE 3

Spectrophotometric vs. biological method of assay of carrots in estimating the vitamin A requirement of human adults.

VITAMIN A REQUIREMENT[1] BASED ON:	SUBJECT							
	6		7		8		9	
	VIT. A IN I.U. PER		VIT. A IN I.U. PER		VIT. A IN I.U. PER		VIT. A IN I.U. PER	
	day	*kg/day*	*day*	*kg/day*	*day*	*kg/day*	*day*	*kg/day*
Spectrophotometric analysis	6645	115	8110	101	5185	90	4450	85
Bioassay	2345	40	2860	36	1770	31	1590	30

[1] Figures are inclusive of small amount of vitamin A present in basal diet.

subjects was derived. Table 4 compares the results from this investigation with findings obtained previously in this laboratory (Booher and Callison, '39; Booher, Callison and Hewston, '39). In these earlier studies a similar method of experimentation was used, the dietary sources of vitamin A being U.S.P. reference cod liver oil no. 1, crystalline carotene in oil, cooked spinach or cooked peas. In these studies also (table 2) chromatographic as well as biological assays were made of the provitamin A active constituents of all the carotene-containing supplements, U.S.P. reference cod liver oil no. 1 with a rated potency of 3000 I.U. per gm being used as the standard of reference.

It has been demonstrated by Callison and Orent-Keiles ('45) and by Porter, Nash, Zscheile and Quackenbush ('46)

TABLE 4

Summary of apparent vitamin A requirements of humans as affected by method of measurement of vitamin A value from different sources.

Subject	Reference cod liver oil, no. 1:	Crystalline Carotene in oil:		Carrots frozen uncooked:		Carrots frozen cooked:		Peas frozen cooked:		Spinach frozen cooked:	
	Assay by Bio.	*Assay by Chem.*	*Bio.*[1]	*Chem.*	*Bio.*[2]	*Chem.*	*Bio.*[2]	*Chem.*	*Bio.*[1]	*Chem.*	*Bio.*[1]
1	36	50	57								
2	56	88	103								
3	52	78	90								
4	25	37	42								
5	34	52	60								
6				115	40	115	40				
7				101	36	101	36				
8				90	31	90	31				
9				85	30	85	30				
10		66	76					42	47		
11		83	96					51	57	68	77
12		92	106							76	87
13										88	101
Average	40.6	68.2	78.8	97.8	34.2	97.8	34.2	46.5	52.0	77.3	88.3

APPARENT HUMAN REQUIREMENTS FOR VITAMIN A DERIVED FROM
(All values expressed in international units per kg body weight)

[1] U.S.P. reference cod liver oil no. 1 used as standard in assay.

[2] S.M.A. β-carotene used as standard in assay.

that U.S.P. reference oils nos. 2 and 3, respectively, are unstable and undependable as standards in vitamin A assays. Although there are less actual direct experimental data to be cited in proof, the stability of U.S.P. reference cod liver oil no. 1 has also been questioned by Morton ('42) and most recently by the United States Bureau of Dairy Industry's Technical Committee in charge of the nation-wide survey ('47) to determine the value of butter as a source of vitamin A in the diet of the people of the United States. Wiseman [4] analyzed immediately upon delivery, a sample of U.S.P. reference cod liver oil no. 1 which had been in transit less than 24 hours and was packed in dry ice during that time. After removal of an aliquot for analysis, the bottle was resealed under nitrogen, stored at reduced temperature and reopened at weekly intervals for a period of 3 weeks, the same precautions being observed at each subsequent resealing and storage. The absorption of the non-saponifiable fraction at 325 mμ was measured by means of a photoelectric spectrophotometer, using a Hilger double monochromator. The values for $E^{1\%}_{1\ cm}$ 325 mμ were 1.50, 1.42, 1.38 and 1.38, respectively. These figures indicate a fall of 8% in the vitamin A potency of the oil during a 3-week period under storage conditions which should keep such losses at a minimum.

In view of these observations on the instability of U.S.P. reference cod liver oil no 1, it is important to reevaluate the bioassay values reported by Booher and Callison ('39) for the crystalline carotene, cooked spinach and cooked peas, in which the standard was U.S.P. reference oil no. 1, and to compare them with the carotene content of these supplements as measured chemically. The bioassay indicated a vitamin A potency for crystalline carotene 15.9% higher than would have been predicted from the carotene analysis, and values 12.9 and 14.7% higher than calculated for the cooked peas and cooked spinach, respectively, assuming complete utilization of carotene from the vegetables (table 2). An uncorrected decrease in the actual potency of the standard of reference employed in a

[4] Wiseman, H. G. Unpublished data.

bioassay leads to just such an apparent increase in the vitamin A value of the material being assayed as reported by Callison and Orent-Keiles ('45). It is, of course, impossible to determine whether or not the entire amount of the discrepancy between the 2 values can be accounted for in this way. Since no spectrophotometric measurements were made of the samples of reference cod liver oil no. 1 used in the assays, the extent of the deterioration of the standard is unknown. However, Callison and Orent-Keiles ('45) have demonstrated that errors of from 30 to 44% can actually occur in vitamin A assays, due to deterioration of U.S.P. reference cod liver oil.

Since U.S.P. reference cod liver oil no. 1 was used in establishing the human requirement for preformed vitamin A by Booher, Callison and Hewston ('39), these values are also probably somewhat too high. On the other hand, the quantitative relationship between the human requirement for preformed vitamin A and for carotene established in this study remains unchanged, since any correction necessitated by the instability of the U.S.P. reference oil no. 1 applies equally to both the cod liver oil and carotene preparations used as supplements. This relationship appears to be remarkably constant for the 5 subjects studied. On the basis of international units per kilogram, subject no. 1 required 62% as much preformed vitamin A as of vitamin A value provided by carotene in oil. Subjects nos. 2, 3, 4, and 5 required 54, 58, 58, and 57% respectively. Since in every case the human "requirement" in terms of the bioassay vitamin A value of the carotene, is greater than the "requirement" in terms of preformed vitamin A, it is apparent that the human being experiences somewhat greater difficulty than the rat in utilizing carotene in this form.

As pointed out above, deterioration in the reference oil may account for the higher values of vitamin A potency obtained by bioassay as compared with chemical analysis of carotene in oil, peas, and spinach. These differences ranged from at least 13 to 16% in magnitude. On the other hand, the lower value obtained for carrots must definitely indicate the

rat's inability to utilize carotene from carrots as effectively as from the other sources. Crystalline β-carotene was the standard used in the vitamin A bioassay of carrots. Therefore the question of instability of reference oil does not enter into consideration of the bioassay figures for this vegetable.

Comparison of the human vitamin A "requirements" calculated from the chemical analyses of the several supplements (table 4) demonstrates that carotene from different sources varies in its availability to the *human* organism. For subjects 10, 11 and 12, carotene from a food source appears to be more fully utilized than carotene in oil. Subjects 10 and 11 required only 63 and 61% as much carotene, respectively, when derived from peas as from crystalline carotene fed in oil. Subjects 11 and 12 required 81 and 83% as much carotene, respectively, when derived from spinach as pure carotene in oil. Unfortunately, it was impossible to study the ability of subjects 6, 7, 8 and 9 to utilize carotene in oil, since the experiments were forced to terminate because the subjects were called to military service.

The rat was able to use carotene from carrots only about one-third as well as from peas, spinach or pure carotene in oil. It is possible that the *human being* utilizes carotene from carrots more efficiently than does the rat. On the basis of the bioassay values, the "requirement" of all 4 subjects for carotene from carrots is below the range found for the 9 subjects receiving carotene from the other sources studied, in all but 1 case (subject 4 for pure carotene). This is true even when the bioassay figures are decreased by 13 to 16% to adjust for possible deterioration of the reference cod liver oil.

In this connection it may be noted, however, that the amount of carotene utilized from carrots by the *human* subjects in this study to support normal dark adaptation is consistent with the recent observation of the Vitamin A Sub-committee of the Accessory Food Factors Committee, Lister Institute and Medical Research Council ('45) that 75% of the carotene

from dietary carrots was eliminated in the feces, leaving 25% available to the body. No details of the chemical methods employed were given in the preliminary report, however.

Inasmuch as neither chemical analyses of carotene content nor bioassays of vitamin A potency are necessarily indicative of the actual availability of this nutrient to the human being, clear-cut differentiation should be made between (1) the vitamin A or carotene content of a food as determined by chemical analysis (2) the vitamin A or carotene requirement of the animal, including man, as determined by the amount of crystalline carotene or vitamin A preparation necessary for the support of a specific physiological function (for example growth or normal dark adaptation), and (3) the efficiency of utilization of the nutrient from a given food to meet these requirements. Graves ('42), Bacharach ('41) and Pyke ('42) have discussed some of the difficulties encountered in interpreting data for practical use and suggestions for overcoming these difficulties have been offered. However, any general factors suggested at this time to be applied as human availability corrections to the vitamin A or carotene values appearing in dietary tables are purely empirical and vary with different foods.

The results of this study emphasize the need for caution when making use of carotene and vitamin A values found in current food composition tables for the planning of diets and evaluation of their adequacy with respect to *human* requirements for this vitamin. In addition, they point to the need for further investigation of vitamin A and carotene utilization by man by means of balance or digestion studies paralleled by similar experiments with rats, since the latter are the standard animals employed for the vitamin A bioassay. It is important to include a large number of human individuals in such a study, since variability in this species is extremely great. Such studies, though difficult and time-consuming, will yield information of value in bringing order out of the confusion which now exists in this field.

ACKNOWLEDGMENTS

The authors wish to express gratitude to the Department of Home Economics of the University of Maryland, College Park, Md., for their generosity in providing a kitchen and dining space and to the 4 students of the University of Maryland who acted as subjects.

We are also indebted to the following members of the staff of the Bureau of Human Nutrition and Home Economics: Miss Ernestine Garofalo for preparation of the meals; to Miss Mary B. Latimer for the carotene determinations; to Miss Mildred Walters for the bioassays; and to Mrs. Alice Harkin for some of the dark adaptation tests.

LITERATURE CITED

BACHARACH, A. L. 1941 Carotene and human requirements. Chem. and Ind., *60:* 435.

BOOHER, L. E., AND E. C. CALLISON 1939 The minimum vitamin A requirements of normal adults. II. The utilization of carotene as affected by certain dietary factors and variations in light exposure. J. Nutrition, *18:* 459.

BOOHER, L. E., E. C. CALLISON AND E. M. HEWSTON 1939 An experimental determination of the minimum vitamin A requirements of normal adults. J. Nutrition, *17:* 317.

CALLISON, E. C., AND E. ORENT-KEILES 1945 Standards in vitamin A assays. U.S.P. reference cod liver oil vs. beta-carotene. Ind. Eng. Chem., Anal. Ed., *17:* 378.

EEKELEN, M. VAN, AND W. PANNEVIS 1938 Absorption of carotinoids from the human intestine. Nature, *141:* 203.

GRAVES, H. C. H. 1942 The vitamin A value of carotene in vegetables. Chem. and Ind., *61:* 8.

KREULA, M., AND A. I. VIRTANEN 1939 Absorption of carotene from carrots in humans. Upsala Läkarefören. Förh., *45:* 355. Cited in Chem. Abst., *34:* column 5897, 1940.

Lister Institute and Medical Research Council, Vitamin A Sub-committee of the Accessory Food Factors Committee 1945 Vitamin A deficiency and the requirements of human adults. Nature, *156:* 11.

MOORE, L. A., AND R. ELY 1941 Extraction of carotene from plant material. A rapid quantitative method. Ind. Eng. Chem., Anal. Ed., *13:* 600.

MORTON, R. A. 1942 Fat-soluble vitamins. Ann. Rev. Biochem., *11:* 365.

PORTER, J. W., H. A. NASH, F. P. ZSCHEILE AND F. W. QUACKENBUSH 1946 Biological inactivity of ζ-carotene. Arch. Biochem., *10:* 261.

PYKE, M. 1942 The vitamin content of vegetables. J. Soc. Chem. Ind., *61:* 149.

Technical Committee in Charge of the Nation-wide Survey 1947 Butter as a source of vitamin A in the diet of the people of the United States. U. S. Dept. Agric., Bureau of Dairy Industry. (In preparation.)

TRESSLER, D. K., AND C. F. EVERS 1943 The Freezing Preservation of Foods, 763 pp. The Avi Pub. Co., Inc., New York.

United States Bureau of Human Nutrition and Home Economics 1944 Family food consumption in the United States, Spring, 1942. U. S. Dept. Agr. Misc. Pub. 550, 157 pp.

United States Bureau of Home Nutrition and Home Economics and National Research Council 1945 Tables of food composition in terms of 11 nutrients. U. S. Dept. Agr. Misc. Pub. 572, 30 pp.

ZSCHEILE, F. P., J. W. WHITE, JR., B. W. BEADLE AND J. R. ROACH 1942 The preparation and absorption spectra of 5 pure carotenoid pigments. Plant Physiol., *17:* 331.

THE ARGININE REQUIREMENT OF YOUNG TURKEY POULTS

F. H. KRATZER, F. H. BIRD, V. S. ASMUNDSON AND S. LEPKOVSKY

Division of Poultry Husbandry, University of California, Davis and Berkeley, California

ONE FIGURE

(Received for publication March 20, 1947)

It has been shown that chicks require arginine for maintenance and growth (Arnold et al., '36; Klose et al., '38; Klose and Almquist, '40). Rats, on the other hand, can synthesize arginine at a rate which permits maintenance but which is insufficient for optimum growth (Burroughs et al., '40). The need for arginine by the turkey poult has not been investigated previously. Three experiments were completed in which young poults were fed an arginine-low ration supplemented with various levels of arginine to determine whether poults require this amino acid, and if they do, the amount necessary for optimum growth.

EXPERIMENTAL

The basal ration used in experiments 1 and 2 contained the following per 100 gm: water-washed casein, 26.6 gm; l(–) cystine, 0.4 gm; glycine, 2.0 gm; calcium gluconate, 5.0 gm; fish oil (3000 A-400 D), 0.5 gm; synthetic vitamins, 0.20591 gm; soybean oil, 3.0 gm; special salt mixture, 1 gm [1]; cholic acid, 0.1 gm; hexane extract of alfalfa, equivalent to 2.0 gm alfalfa; folic acid equivalent to 4 gm solubilized liver; and glucose to make 100 gm. The special salt mixture contained (in %) 96.47

[1] Composition: tricalcium phosphate, 3.5 gm; dipotassium phosphate, 1.29 gm; potassium chloride, 0.3 gm; magnesium sulfate, 0.4 gm; sodium silicate, 0.2 gm.

sodium chloride, 0.49 manganese, 0.1 iron, 0.05 copper, 0.05 zinc, 0.05 aluminum, 0.002 cobalt and 0.04 iodine. The synthetic vitamins included choline chloride 0.2 gm, biotin 0.00001 gm, alpha tocopherol 0.001 gm, thiamine 0.0005 gm, riboflavin 0.0005 gm, pyridoxine 0.0004 gm, d-pantothenic acid 0.002 gm, and nicotinic acid 0.0015 gm. For the third experiment the basal ration was essentially the same as in the previous tests, but the casein was increased to 28% and pteroylglutamic acid [2] was substituted for the folic acid concentrate. The l(+) arginine monohydrochloride, l(−)cystine, and glycine used were commercial preparations.

Bronze turkey poults were used in these experiments. At hatching they were placed in electrically heated batteries with raised wire floors and fed a regular starter mash. At 1 week of age, the poults were divided into equivalent weight groups of 9 poults each in the first 2 experiments and 8 each in the third test. The experimental diets and water were supplied *ad libitum*. The first and second experiments were continued for 10 days each and the third experiment for 12 days.

RESULTS AND DISCUSSION

Block and Bolling ('45) have reported that casein contains 4.1% arginine on a 16% nitrogen basis. Since the washed casein in the present series of experiments contained 82.5% crude protein, it was assumed that it contained 3.4% arginine. This value was used throughout in calculating the arginine content of the rations.

Data for the 3 experiments are shown in figure 1. The percentage gains per day were calculated according to the following formula:

$$\text{per cent gain per day} = \frac{\text{average gain} \times 100}{\text{average body weight} \times \text{number of days}}$$

The results agree well with those to be expected except for the groups in experiments 1 and 2 which were fed 1.15%

[2] The pteroylglutamic acid was kindly supplied by Lederle Laboratories, Inc., Pearl River, N. Y., through the courtesy of Dr. T. H. Jukes.

arginine: these gained less than the groups fed slightly less (1.07%) arginine. In each of the first 2 experiments the highest level of dietary arginine produced the greatest gain, with a greater gain on the 1.31% than on the 1.23% level (fig. 1). These results indicated that the optimum level had not been exceeded but since the gains made by the poults fed the highest level of arginine were only slightly less than the maximum gains expected on this type of diet, the optimum level could not be greatly in excess of 1.31%. To determine this optimum level, the amount of arginine was increased to

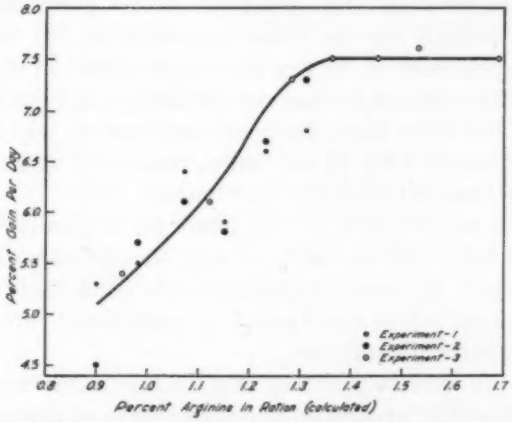

Fig. 1 The relation of gain in weight of young poults to the level of arginine in their ration.

1.69% of the ration in the third experiment. The growth curve showed a plateau at a level of approximately 1.35% of arginine.

Mortality did not seem to be related to the level of arginine fed, with the possible exception of the first test in which 2 poults died in the group receiving the lowest level of arginine, while 1 poult died in the group receiving 1.07% arginine. In the second test 2 poults died in each of the groups receiving 0.98 and 1.31% arginine and 1 poult in each of the groups receiving 0.90, 1.07 and 1.23% arginine. In the third trial,

the only poult that died was in the group receiving 1.12% arginine.

The poult requirement of approximately 1.35% arginine, as indicated in figure 1, is considerably greater than the chick requirement which has been reported to be 0.9% of the ration (Almquist, '45). The poult requirement for total protein is approximately 1.2 (i.e., 24/20) times greater than the chick requirement. If the chick requirement for arginine were increased by this factor it would equal 1.08% arginine which is still only 80% of the amount required for poults. Thus the high requirement for arginine by the poult cannot be explained entirely by the higher requirement for total protein. The difference in the arginine requirement of chicks and poults is quite similar to that reported previously to exist for lysine. In the latter case, the poult requirement was found to be approximately 1.3% of the ration, compared with 0.9% for the chick (Grau, Kratzer and Asmundson, '46).

The high requirement of the poult for arginine indicates that it has very limited ability, if any, to synthesize arginine. In this respect the poult is similar to the chick and is in contrast to the rat which can grow at a suboptimal rate without a dietary source of arginine.

It is of interest to mention that in the third experiment, gains produced in groups on adequate levels of arginine were slightly greater than gains produced in a control group fed a practical poult starter.

SUMMARY

Poults were fed a purified type ration with casein, glycine, cystine, and various levels of arginine as the only source of amino acids. Approximately 1.35% arginine was required in the total ration to produce optimum growth.

LITERATURE CITED

ALMQUIST, H. J. 1945 Effective use of proteins in the nutrition of the chick. Transaction, Am. Assoc. of Cereal Chemists, *3:* 158.

ARNOLD, A., O. L. KLINE, C. A. ELVEHJEM, AND E. B. HART 1936 Further studies on the growth factor required by chicks, the essential nature of arginine. J. Biol. Chem., *116:* 699.

BLOCK, R. J., AND D. B. BOLLING 1945 The Amino Acid Composition of Proteins and Foods. Charles C. Thomas, Springfield, Ill. (publisher).

BURROUGHS, E. W., H. S. BURROUGHS AND H. H. MITCHELL 1940 The amino acids required for the complete replacement of endogenous losses in the adult rat. J. Nutrition, *19:* 363.

GRAU, C. R., F. H. KRATZER AND V. S. ASMUNDSON 1946 The lysine requirements of poults and chicks. Poultry Sci., *25:* 529.

KLOSE, A. A., AND H. J. ALMQUIST 1940 The ability of citrulline to replace arginine in the diet of the chick. J. Biol. Chem., *135:* 153.

KLOSE, A. A., E. L. R. STOKSTAD AND H. J. ALMQUIST 1938 The essential nature of arginine in the diet of the chick. J. Biol. Chem., *123:* 691.

FOLIC ACID, PARA-AMINOBENZOIC ACID AND ANTI-PERNICIOUS ANEMIA LIVER EXTRACT IN SWINE NUTRITION.[1]

T. J. CUNHA, L. K. BUSTAD, W. E. HAM, D. R. CORDY, E. C. McCULLOCH, I. F. WOODS, G. H. CONNER AND M. A. McGREGOR

Divisions of Animal Husbandry, Veterinary Science and Home Economics, State College of Washington, Pullman

(Received for publication March 1, 1947)

Lindley and Cunha ('46) showed that the addition of inositol and biotin to the ration (purified) of the young pig did not increase growth or efficiency of feed utilization. However, they showed that biotin and inositol were needed by the pig when sulfathalidine was included in the ration at a 0.5% level. In their work with the pig it was postulated that inositol functioned indirectly by stimulating intestinal synthesis of biotin. A biotin deficiency syndrome similar to that observed by Lindley and Cunha ('46) as a result of feeding sulfathalidine in the ration was shown to occur when desiccated egg white, at a 30% level, was included in the ration of the pig (Cunha, Lindley and Ensminger, '46). Cartwright, Wintrobe and Humphreys ('46) produced an anemia in a pig, by including 2% of sulfasuxidine in the ration, which responded to a highly purified liver extract; however, they were not sure whether the anemia was due to a folic acid deficiency.

The experiment which is herein reported was undertaken to determine whether the addition of folic acid and para-

[1] Published as Scientific Paper no. 706, College of Agriculture and Agricultural Experiment Stations, State College of Washington, Pullman. This study was supported in part by a grant from the Nutrition Foundation, Inc., New York, N. Y.

aminobenzoic acid alone, or in combination with inositol and biotin, would be of any benefit, when added to a purified basal ration containing the 6 B-complex vitamins: thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline. In addition, a liver preparation (used in pernicious anemia in humans) was compared with folic acid for the pig. The effect of sulfathalidine administration on vitamin requirements of the pig was also studied.

EXPERIMENTAL

Eight-week old pigs were used in the lots where purified rations were fed. These pigs were farrowed by 4 Chester White gilts which had been bred to a Danish Landrace boar. The dams of these pigs were fed a ration of the following percentage composition during gestation and lactation: wheat 27, barley 27, cull peas 20, millrun 10, tankage 5, alfalfa meal 10, oyster shell 0.5, and iodized salt 0.5%.

The pigs were distributed equally into lots on the basis of weight, sex, and age. One pig from each of the 4 dams was placed in each of the 5 lots where purified rations were fed. The rations fed are shown in tables 1 and 2. The same pigs were used throughout the 2 phases of this experiment. During the first phase (7-week period) the effect on the nutrition of the pig of folic acid and para-aminobenzoic acid, alone and in combination with inositol, biotin, and a liver preparation [2] used in the treatment of pernicious anemia in humans, was investigated. During the second phase (5-week period) the effect of sulfathalidine on the need for these vitamins by the pig was determined.

The purified basal ration used in this experiment was the same as that reported previously by Heinemann, Ensminger, Cunha and McCulloch ('46). The ration consisted of casein [3] 26.1%, sucrose 57.7%, lard 11% and mineral mix [4] 5.2%.

[2] "Concentrated Solution Liver Extract, Parenteral" used for treatment of pernicious anemia. This was supplied by Lederle Laboratories, Pearl River, N. Y.

[3] Vitamin Test Casein GBI, manufactured by General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Same as used by Wintrobe ('39), and as modified by Heinemann et al. ('46).

Water soluble vitamins were fed (in mg per kg of live weight daily) as follows: thiamine 0.52, riboflavin 0.12, niacin 1.20, pantothenic acid 0.50, pyridoxine 0.20, and choline chloride 10.00; fat soluble vitamins were supplied (per pig daily) as follows: vitamin A, 5,000 I.U., vitamin E, 57 mg, vitamin K, 2 mg, vitamin C, 50 mg, and vitamin D, 700 I.U.

A modification of the "paired feeding technique" (Mitchell and Beadles, '30), was used in order to maintain the same feed intake by all pigs fed purified rations. Thus, the feed intake of the pigs was limited to the amount consumed by the pig with the least appetite — with the vitamins and the sulfonamide studied being the only variable among the lots of pigs. The pigs were fed in individual feeding stalls. To prevent coprophagy the pigs were kept on raised floors which were washed twice daily. At no time during the experiment was any tendency for the pigs to eat their feces observed.

To prevent rancidity and subsequent destruction of vitamins, the purified ration was mixed every third day and kept in an ice box. All vitamin solutions were stored under refrigeration. The required amount of the vitamins fed each pig was measured in calibrated pipettes and placed on top of the ration every other day (every fourth feeding) just before feeding time.

The pigs in lots VI and VII were fed natural grain rations. The pigs in lot VIA were of similar background and age as those fed purified rations. The rest of the pigs fed natural grain rations were 1 week younger and from Chester White sows fed the same ration as that fed the dams of the pigs on purified rations.

In lot VI, the natural (fish meal) ration consisted of wheat 20, barley 18.5, millrun 4.0, cull peas 16.0, soybean oil 6.0, fish meal (66.8% protein) 30.0, alfalfa meal (sun-cured) 5.0, and salt 0.5%. In lot VIA the pigs were self-fed this ration, whereas, in lot VIB the intake of feed was limited.

In lot VII, the natural (tankage) ration consisted of wheat 46.0, barley 35.0, tankage (dry rendered — 56.8% protein) 13.5, alfalfa meal (sun-cured) 5.0, and salt 0.5%. The amount

of feed fed these 6 pigs was the same and limited to the amount eaten by the pig with the least appetite. One-half of the pigs (those in lot VIIA) were given folic acid.

Each pig was bled weekly and blood was taken from the marginal ear vein for determination of hemoglobin, erythrocyte, leucocyte, and differential cell counts as well as reticulocyte studies.

Pipettes were filled from the flowing blood on the ear. Smears were prepared from the same source, the stagnant blood being wiped away as it gathered. Hemoglobin was determined according to the method of Evelyn ('36). Leucocytes were counted in 2% acetic acid diluent tinted with gentian violet. Erythrocytes were diluted with Hayem's solution. Smears for differential leucocyte counts were stained with Wright's stain and 200 cells were examined. Reticulocyte smears were stained with brilliant cresyl blue and counterstained with Wright's stain.

RESULTS AND DISCUSSION

Growth studies

The data in table 1 show that no appreciable beneficial effect on external appearance, growth, or efficiency of feed utilization was obtained when either folic acid or para-aminobenzoic acid was added alone or in combination with inositol and biotin to the purified basal ration. One pig (no. 330) which received the anti-pernicious anemia liver extract in addition to folic acid (lot 1B) made poor gains, became rough and scaly, and developed a brownish exudate throughout the surface of the body. Evidently, this pig (no. 330) reacted abnormally by developing an allergy-like condition in response to something present in the liver extract.[5]

Frequent scouring, during the first 5 weeks, was observed in the pigs on a restricted intake of the natural ration containing fish meal (lot VIB). The scouring accounts for the

[5] Dr. J. L. Rice, of Lederle Laboratories, saw this pig and stated that occasionally humans will react in a similar manner to the liver extract.

TABLE 1

The effect of folic acid (FA), p-aminobenzoic acid (PAB), inositol (IN), biotin (B), and anti-pernicious anemia liver extract (LE) on growth.

Type of ration	PURIFIED						NATURAL GRAIN			
							30% fish meal in the ration		13.5% tankage in the ration	
Ration fed	Basal [1] only	Basal + FA + LE [2]	Basal + FA [3]	Basal + PAB [4]	Basal + PAB + IN [5] + B [6]	Basal + PAB + IN + B + FA	Ration self-fed	Amount of ration fed was limited	Folic acid added	Nothing added
Lot number	IA	IB	II	III	IV	V	VIA	VIB	VIIA	VIIB
Number of pigs	4	2	4	4	4	4	3	3	3	3
Initial age, weeks	8	8	8	8	8	8	8	7	7	7
Number of weeks on trial	7	7	7	7	7	7	7	7	4	4
Av. initial weight, lbs.	34.7	30.5	32.7	33.5	32.5	32.0	42	31.3	36.6	39.6
Av. daily gain, lbs.	0.78	0.74	0.80	0.80	0.86	0.82	0.92	0.61	0.75	0.58
Av. daily feed consumption, lbs.	1.63	1.63	1.63	1.63	1.63	1.63	2.66	1.91	2.32	2.32
Lbs. of feed per lb. of gain	2.08	2.20	2.03	2.03	1.89	1.98	2.89	3.13	3.09	4.00

[1] Basal ration contained 6 B-complex vitamins (thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline).

[2] Injected intramuscularly, in the gluteal muscle, every fourth day. It was injected at a rate of 0.067 ml daily (1 U.S.P. injectable unit) during the first 3 weeks. Following that period, the amount injected was doubled.

[3] Crystalline folic acid was fed in all lots at a level of 50 μg per 100 gm of feed.

[4] Para-aminobenzoic acid was fed in all lots at a level of 10 mg per 100 gm of feed.

[5] Inositol was fed in all lots at a level of 100 mg per 100 gm of feed.

[6] Biotin was fed in all lots at a level of 20 μg per 100 gm of feed.

poor gains and lowered efficiency of feed utilization obtained when comparing results on self-feeding versus limited feeding of the ration containing fish meal during the first 7 weeks of the experiment.

Supplementing with folic acid the natural ration containing tankage resulted in faster gains and in less feed being required per pound of gain. However, this effect was obtained only during the first 4 weeks of the experiment. In the next 5 weeks (table 2), folic acid did not improve the rate of gain. However, at the end of the 9-week feeding trial, the pigs fed folic acid were cleaner in appearance and had bigger appetites. It is possible that if the pigs fed folic acid had not been restricted in their feed intake, they would have eaten more and consequently outgained the pigs not being fed this dietary factor.

Occasional scouring, characterized by grayish feces, was obtained in all lots of pigs fed the purified rations during the first 7 weeks of the experiment. The scouring usually lasted from 36 to 48 hours and did not seem to have any effect on appetite. This scouring was observed even though, in lot V, all the known vitamins were being fed. No scouring was obtained with the pigs during the second 5 weeks of the experiment when sulfathalidine was included in the ration. However, the pigs fed the basal ration without sulfathalidine, continued to scour occasionally during the second 5 weeks of the experiment. Therefore sulfathalidine, fed at a level of approximately 0.65 gm per 10 pounds body weight, was effective in preventing a type of scours which occurred even when all known vitamins were included in the ration. Ferrin ('46) found that oral administration of sulfathalidine at a rate of 0.75 gm per 10 pounds of body weight was effective in stopping scours which developed in pigs fed methionine in addition to a natural ration. Scours in young pigs fed natural rations have been reported by Ross et al. ('44), Fairbanks et al. ('44) and Whitehair et al. ('46). Whether there is any relation between the scours observed by these workers and

TABLE 2

The effect of sulfathalidine (ST) on the response of the pig to folic acid (FA), p-aminobenzoic acid (PAB) inositol (IN), biotin (B), and anti-pernicious anemia liver extract (LE).

Type of ration	PURIFIED								NATURAL GRAIN			
									30% fish meal in the ration		13.5% tankage in the ration	
Previous ration (table 1)	Basal	Basal	Basal + LE + FA	Basal + FA	Basal + PAB	Basal + PAB + IN + B		Basal + PAB + IN + B + FA	Ration self-fed	Amt. of ration fed was limited	Folic acid added	Nothing added
Ration fed	Basal	Basal + ST [1]	Basal + LE + FA [2] + ST	Basal + FA + ST	Basal + PAB + ST	Basal + PAB [3] + IN [3] + B [3] + ST	Basal + PAB + IN + B + ST + LE	Basal + PAB + IN + B + ST + FA	Same as above	Same as above	Same as above	Same as above
Lot number	IA	IAA	IB	II	III	IVA	IVB	V	VIA	VIB	VIIA	VIIB
Number of pigs	2	2	2	4	4	2	2	4	3	3	3	3
Initial age, weeks	15	15	15	15	15	15	15	15	15	14	11	11
Number of weeks on trial	5	5	5	5	5	5	5	5	5	5	5	5
Av. initial weight, lbs.	74.0	72.5	75.5	72.0	72.5	75.0	74.0	72.2	86.3	61.0	57.3	56.0
Av. daily gain, lbs.	1.47	1.44	1.19	1.46	1.39	1.42	1.44	1.43	1.43	1.07	0.86	0.87
Av. daily feed consumption, lbs.	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	6.07	3.33	3.19	3.19
Lbs. of feed per lb. of gain	1.93	1.96	2.37	1.94	2.03	1.99	1.96	1.97	4.24	3.11	3.70	3.66

[1] Sulfathalidine was fed at a level replacing 0.5% of the ration.

[2] Folic acid was fed in all lots at a level of 100 μg per 100 gm of ration during last 4 weeks. During first week it was fed at level of 50 μg per 100 gm of ration.

[3] Biotin, inositol, and para-aminobenzoic acid were fed at same levels indicated in table 1.

that obtained in this work with purified rations is difficult to say.

A small amount of blood was frequently noted in the feces of the pigs fed purified rations. Wood in the feces, as a result of fence chewing, was found with all the pigs fed purified rations. No fence chewing or blood in the feces was observed with the pigs fed the natural grain rations. In an effort to test whether a lack of sufficient phosphorus might be responsible for the fence chewing, an extra 10 gm of $CaHPO_4$ were given each pig daily for 10 days. This was followed by increasing the total mineral intake of the ration from 5.2 to 7.2% for 21 days. The addition of the extra minerals seemed to alleviate, to a small extent, the fence chewing but did not eliminate it. Fence chewing has consistently been observed at this station with young pigs fed purified rations.

Since the addition of either folic acid or para-aminobenzoic acid to the basal ration for a 7-week period was of no benefit on gains or efficiency of feed utilization, it was assumed that possibly the pig may be able, through the action of intestinal bacteria, to synthesize enough of these 2 vitamins for those needs. However, there is the possibility that enough of these 2 vitamins may have been stored during the suckling period of these pigs to last them through this experiment. In order to test whether these 2 vitamins were being synthesized, sulfathalidine which has been used to inhibit the growth of intestinal bacteria and hence vitamin synthesis with the pig (Lindley and Cunha, '46) was used.

The data in table 2 show that the addition of sulfathalidine to the purified basal ration for a 5-week period had no effect on the need of the pig for either folic acid or para-aminobenzoic acid as measured by the rate of gain and efficiency of feed utilization. Among all the lots of pigs receiving purified rations, there was little or no difference in gains except in lot 1B where the anti-pernicious anemia liver preparation was fed in addition to folic acid. In this lot, 1 pig (no. 330) accounted for the poor gains. The other pig in this lot gained as well as the pigs in the other lots fed purified rations. The

exudate which pig no. 330 exhibited at the end of the experiment was not as severe as it was at the end of the first 7 weeks. Pig no. 330 developed cracked feet by the end of the experiment which were typical of those observed on a biotin deficiency with the pig by Lindley and Cunha ('46) and Cunha et al. ('46). After the third week, pig no. 330 began losing some hair on the posterior part of the ham and displaying a sensitivity in the feet and a tendency to lie down and remain inactive. All 4 feet became cracked and bled occasionally. The pig also displayed considerable hypersensitivity when forced to move. The hair loss and cracked feet are typical of a biotin deficiency. The other pig in this lot also had 4 cracked feet and a slight hair loss on the posterior part of the ham. However, the most marked biotin deficiency symptoms were obtained with pig no. 330.

In lot IAA (basal + sulfathalidine) 1 pig displayed cracks in the feet typical of a biotin deficiency. The other pig in this lot had a slight hair loss on the posterior part of the ham. In lot II (basal + folic acid) all 4 pigs displayed slight to moderately cracked feet typical of a biotin deficiency. Two pigs displayed a slight hair loss on the posterior part of the ham. In lot III (basal + PAB) 2 pigs displayed moderately cracked feet and 1 of these pigs had a slight hair loss on the posterior part of the ham. All pigs in lots IA, IVA, IVB, and V were normal in appearance with regard to hair coat and feet. This substantiates the work of Lindley and Cunha ('46), which showed that biotin in the ration would prevent the symptoms discussed above. The biotin deficiency symptoms exhibited in this work were very mild as compared to those observed previously by Lindley and Cunha ('46). This may be due to 2 things: (1) In the previous work lighter pigs were used and sulfathalidine was fed after the pigs had previously been fed sulfaguanidine for 5 weeks, and (2) the pigs were fed sulfathalidine for 6 weeks in the previous work as compared to 5 weeks in this trial. Miller ('45) showed that sulfathalidine fed to rats over a long period of time caused nutritional deficiency symptoms which were corrected by the feeding of

biotin and folic acid. Cartwright, Wintrobe and Humphreys ('46) were able to produce an anemia in the pig which responded to a highly purified liver extract. However, the anemia was produced after feeding 2% of sulfasuxidine in the ration for an extended period of time; they were not sure that the anemia was due to a folic acid deficiency.

The data in table 2 show that the pigs fed a limited amount of the natural ration containing fish meal were very efficient in utilizing their feed. During the second 5-week period, the pigs fed a limited amount of the fish meal ration required 3.11 pounds of feed per pound of gain whereas the pigs self-fed the same ration required 4.24 pounds of feed per pound of gain.

Studies of the blood

Hemoglobin. The data in table 3 show that during the first 7-week period the addition of folic acid to the basal purified ration was of some help in hemoglobin formation. The addition of para-aminobenzoic acid to the basal ration resulted in slightly higher hemoglobin values. Those obtained with the pigs fed the natural grain rations (lots VI and VII) are somewhat in line with others reported in the literature by Kernkamp ('32), Croft and Moe ('32) and Nordskog et al. ('44).

It is of interest to note that the addition of folic acid to the basal purified ration resulted in higher hemoglobin values than those obtained with the pigs fed the natural grain rations. Since the natural ration containing 30% fish meal may be considered very well balanced nutritionally, one might wonder whether many supposedly well-balanced natural grain rations really support maximum hemoglobin formation. The question might also be raised as to whether the pigs fed the basal ration plus folic acid were better off nutritionally since their hemoglobin values were a little higher than those of the pigs self-fed the natural ration containing 30% fish meal.

The addition of folic acid alone to the basal purified ration (lot II) stimulated hemoglobin formation. However, when folic acid was added along with the liver extract (lot IB) or

other vitamins (lot V), it did not stimulate hemoglobin formation. This finding is difficult to explain. It is possible that it may have been caused by some vitamin imbalance. Suggestions of a possible imbalance of vitamins were obtained by Cunha et al. ('43) with rats, and by Cunha et al.

TABLE 3

The effect of folic acid (FA), p-aminobenzoic acid (PAB), inositol (IN), biotin (B), and anti-pernicious anemia liver extract (LE) on hemoglobin level of pigs.

LOT NO.	RATION FED		BLOOD HEMOGLOBIN [1] (GM/100 ML)		
			First 7 weeks	Next 5 weeks	
			No sulfonamide added	No sulfonamide added	Sulfathalidine added
IA	Basal		12.51	12.89	13.60
IB	Basal + FA + LE		12.33		13.23
II	Basal + FA		14.18		13.90
III	Basal + PAB		13.49		13.01
IVA	Basal + PAB + IN + B		12.77		13.40
IVB	Basal + PAB + IN + B + LE				13.24
V	Basal + PAB + IN + B + FA		12.17		13.09
VIA	Natural ration with fish meal	Self-fed	12.57	13.05	
VIB		Limited amount fed	12.52	13.13	
VIIA	Natural ration with tankage	Folic acid added	11.47	11.32	
VIIB		Nothing added	11.55	11.54	

[1] The hemoglobin values are an average figure of all the times the pigs were bled.

('44) with sows. With both rats and sows, there were many instances where the addition of 1 vitamin or a combination of vitamins gave poorer results than when no vitamins were added to the basal ration being studied.

Of interest is the fact that the hemoglobin values were about the same with the pig self-fed as compared with those fed a limited amount of the same natural grain ration containing fish meal. The addition of folic acid to the natural grain ration containing tankage had no effect in stimulating hemoglobin formation although it stimulated growth to a slight extent during the first 4 weeks of the experiment. The addition of sulfathalidine to the purified rations did not have any effect in lowering hemoglobin values. This may have been caused by too low a level being fed, or the feeding of sulfathalidine not being continued for a long enough period.

Erythrocytes. Weekly averages of all groups of pigs showed erythrocyte counts in the range of 6 to 10 millions per mm^3. Kernkamp ('32) reported an average of about 7 million erythrocytes in pigs 40 to 80 days of age. Figures reported by Kolmer and Boerner ('41) show a range of 5 to 9 million erythrocytes per mm^3 for swine, although the ages of their pigs were not given. It would thus appear that neither anemia nor polycythemia was produced in pigs in this experiment since their erythrocyte counts were within normal ranges. No appreciable differences in number of erythrocytes were obtained between the various groups of pigs. Excessive numbers of immature or abnormal erythrocytes were not observed.

Reticulocytes. The reticulocyte counts in the various groups of pigs averaged from 0.3–2.6% of the erythrocytes. The reticulocyte numbers were consistently lower during the period sulfathalidine was fed than they were in the same group during the first part of the experiment. Coffin ('46) reports a reticulocyte range of 0.5–4.5% in swine. It is assumed that the reticulocyte counts in this experiment were within a normal range.

Leucocytes. Weekly averages of all groups of pigs showed leucocyte counts in the range of 10,000 to 30,000 per mm^3 Kernkamp ('32) reported an average leucocyte count of about 21,600 in pigs 54 to 80 days old. From Kernkamp's work it would appear that young pigs have a relatively high leucocyte count, as was found in this work. While some of the leucocyte counts were high, there was no appreciable difference among the various groups, and the counts were regarded as being within normal range.

During the last 3 or 4 weeks of the experiment there developed a moderate relative lymphocytosis and neutropenia, which was less marked with the natural grain ration fed the pigs in lots VI and VII. It may be that this difference was due to some factor or factors contained in the natural rations and not contained in the purified rations. There was no appreciable difference among the groups of pigs fed purified rations in this regard.

SUMMARY AND CONCLUSIONS

During a 7-week trial, no beneficial effect on external appearance, growth, or efficiency of feed utilization was obtained when either folic acid or para-aminobenzoic acid was added alone or in combination with inositol and biotin to a purified basal ration which contained the 6 B-complex vitamins — thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline.

The addition of folic acid or para-aminobenzoic acid alone to the basal purified ration stimulated hemoglobin formation to a small extent. However, when folic acid was added to the purified basal ration in addition to the liver extract or other vitamins, it did not stimulate hemoglobin formation. It is possible that this may have been due to some vitamin imbalance. The addition of folic acid alone to the basal purified ration resulted in higher hemoglobin values than those obtained with pigs fed a natural grain ration containing 30% fish meal. The provision of folic acid to pigs fed a natural grain ration containing tankage did not have any effect on hemoglobin

formation although it caused some stimulation of growth during the first 4 weeks and caused the pigs to be cleaner in appearance and have bigger appetites at the end of the experimental period. This may also mean that even though folic acid does not help growth with a purified ration, it may, under certain conditions, promote growth with natural grain rations.

One pig, being injected with the anti-pernicious anemia liver extract, reacted to it abnormally. The pig made poor gains, developed rough and scaly skin, and a brownish exudate throughout the surface of the body.

Sulfathalidine, fed at a level of approximately 0.65 gm per 10 pounds of body weight, was effective in preventing the scours, characterized by a grayish feces, which occurred periodically with the pigs fed purified rations even in the group where all the known vitamins were fed.

ACKNOWLEDGMENTS

We are indebted to Dr. D. F. Green and Merck and Company, Rahway, N. J., for supplying biotin and other B-complex vitamins. The cooperation of Dr. T. H. Jukes and Lederle Laboratories, Pearl River, N. Y., in supplying pteroylglutamic acid and the anti-pernicious anemia liver extract is gratefully appreciated. The sulfathalidine used in this experiment was obtained through the courtesy of Dr. S. F. Scheidy, Sharp and Dohme, Glenolden, Pa. The interest and suggestions given by Dr. C. G. King, Nutrition Foundation, Inc., N. Y., are gratefully acknowledged.

LITERATURE CITED

CARTWRIGHT, G. E., M. M. WINTROBE AND S. HUMPHREYS 1946 Production of amenia in a pig which responds to purified liver extract. J. Lab. Clin. Med., *31:* 423.

COFFIN, D. L. 1946 Manual of Veterinary Clinical Pathology. Comstock Publishing Co., Inc., Ithaca, N. Y., p. 126.

CRAFT, W. A., AND L. H. MOE 1932 Statistical observations involving weight, hemoglobin, and the proportion of white blood cells in pigs. J. Am. Vet. Med. Asso., *34:* 405.

CUNHA, T. J., S. KIRKWOOD, P. H. PHILLIPS AND G. BOHSTEDT 1943 Effect of inositol upon rat alopecia. Proc. Soc. Exp. Biol. Med., *54:* 236.

CUNHA, T. J., D. C. LINDLEY AND M. E. ENSMINGER 1946 Biotin deficiency syndrome in pigs fed desiccated egg white. J. Animal Sci., *5:* 219.

CUNHA, T. J., O. B. ROSS, P. H. PHILLIPS AND G. BOHSTEDT 1944 Further observations on the dietary insufficiency of a corn-soybean ration for reproduction in swine. J. Animal Sci., *3:* 415.

EVELYN, K. A. 1936 Stabilized photelectric colorimeter with light filters. J. Biol. Chem., *115:* 63.

FAIRBANKS, B. W., J. L. KRIDER AND W. E. CARROLL 1944 Distillers by-products in swine rations. I. Creep-feeding and growing-fattening rations. J. Animal Sci., *3:* 29.

FERRIN, E. F. 1946 The addition of synthetic nutrients to protein supplemental feeds in swine rations. J. Animal Sci., *5:* 42.

HEINEMANN, W. W., M. E. ENSMINGER, T. J. CUNHA AND E. C. MCCULLOCH 1946 The relation of the amount of thiamine in the ration of the hog to the thiamine and riboflavin content of the tissue. J. Nutrition, *31:* 107.

KERNKAMP, H. C. H. 1932 The blood picture of pigs kept under conditions favorable to the production and to the prevention of so-called anemia of suckling pigs. Minnesota Agr. Exp. Sta. Tech., Bull. 86, p. 32.

LINDLEY, D. C., AND T. J. CUNHA 1946 Nutritional significance of inositol and biotin for the pig. J. Nutrition, *32:* 47.

MILLER, A. K. 1945 The effect of succinylsulfathiazole and phthalysulfathiazole on the bacterial flora of rat feces. J. Nutrition, *29:* 143.

MITCHELL, H. H., AND JESSIE R. BEADLES 1930 The paired-feeding method in nutrition experiments and its application to the problem of cystine deficiencies in food proteins. J. Nutrition, *2:* 225.

NORDSKOG, A. W., R. E. COMSTOCK AND L. M. WINTERS 1944 The relationship between certain blood components and rate of growth in swine. J. Animal Sci., *3:* 422.

ROSS, O. B., P. H. PHILLIPS, G. BOHSTEDT AND T. J. CUNHA 1944 Congenital malformations, syndactylism, talipes, and paralysis agitans of nutritional origin in swine. J. Animal Sci., *3:* 406.

WHITEHAIR, C. K., R. H. GRUMMER, G. BOHSTEDT AND P. H. PHILLIPS 1946 Effect of vitamin supplementation and sulfa drug therapy on gray scours in small pigs. J. Animal Sci., *5:* 418.

WINTROBE, M. M. 1939 Nutritive requirements of young pigs. Am. J. Physiol., *126:* 375.

THE SPARING ACTION OF PROTEIN ON THE PANTOTHENIC ACID REQUIREMENT OF THE RAT

II. URINARY AND FECAL EXCRETION OF PANTOTHENIC ACID [1]

MARJORIE M. NELSON,[2] FREEKE VAN NOUHUYS AND HERBERT M. EVANS

Institute of Experimental Biology, University of California, Berkeley

TWO FIGURES

(Received for publication March 5, 1947)

The sparing action of high protein (casein) diets on the pantothenic acid requirement of the rat (Nelson and Evans, '45) was shown by the increased growth and survival of rats deficient in pantothenic acid from birth or from weaning. This sparing action may be due to (1) an "absolute" decrease in the requirement for the vitamin; (2) a relative decrease because of decreased vitamin loss by urinary excretion; or (3) an increased intestinal synthesis of the vitamin. To investigate the latter 2 possibilities the urinary and fecal excretion of pantothenic acid has been determined for deficient rats maintained on purified diets varying from 24% to 64% casein.

The present study shows that the fecal excretion of the vitamin was variable and a relation to the dietary protein level not apparent. This would seem to exclude the third view that increased intestinal synthesis of the vitamin occurs. On the

[1] Aided by grants from the Board of Research and from the Department of Agriculture of the University of California, and the Rockefeller Foundation, New York City. The following materials were generously contributed: crystalline B vitamins from Hoffmann-LaRoche Co., Nutley, N. J., and Merck and Co., Inc., Rahway, N. J.; alpha-tocopherol from Merck and Co., Inc.

[2] General Mills Fellow.

other hand, the urinary vitamin excretion was definitely observed to increase with the protein intake. The second explanation that a decreased vitamin loss by urinary excretion occurs is, therefore, erroneous. The data here presented, therefore, indicate instead that there is an unexplained "absolute" decrease in the rat's requirement for pantothenic acid as such when higher protein intake occurs.

The marked increases observed in urinary vitamin excretion prompted a reexamination of the pantothenic acid content of various purified and "vitamin-free" caseins, all of which were found to contain significant amounts of pantothenic acid. When the calculated amount of pantothenic acid present in the high-protein diets was added to the high-carbohydrate diet, such supplemented diets were still not equal in sparing action to the high-protein diets on which littermates were maintained.

EXPERIMENTAL PROCEDURES

Twenty-one day old rats, littermates of the Long-Evans strain, were divided into equivalent groups, balanced according to body weight, and placed in individual cages with screens. Males were used for all experiments except the first one. The animals were examined and weighed every 5 days; those in poor condition were checked daily.

The purified pantothenic acid-deficient diets used varied only in their proportions of carbohydrate and protein. The basal diet (high-carbohydrate) contained 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil,[3] and 4% salts.[4] The high-protein diet contained 64% casein and 24% sucrose with the remainder of the constituents identical. Both diets contained the following crystalline B vitamins per kilogram of diet: 5 mg thiamine-HCl, 5 mg pyridoxine-HCl, 10 mg riboflavin, 10 mg p-aminobenzoic acid, 20 mg nicotinic acid, 400 mg inositol, and 1 gm choline chloride. Each rat received weekly a fat-soluble vitamin mixture con-

[3] Crisco.

[4] Salts no. 4 of Hegsted et al. ('41).

taining 325 mg corn oil,[5] 400 U.S.P. units vitamin A, 58 Chick Units vitamin D, and 3 mg alpha-tocopherol.

The microbiological assay method of Strong et al. ('41) as modified by Neal and Strong ('43) and by Strong ('44) was used for pantothenic acid analyses. Urine and feces were collected for a 24-hour period and pooled for each dietary group. Individual metabolism cages were used for each rat and every precaution taken to eliminate contamination of urine with the feces. This was especially important because the pantothenic acid content of feces was considerably higher than that of the urine. Food was not given to the rats during the 24-hour collection period because of the difficulty in preventing scattering of the diet with the subsequent contamination of urine and feces. Also, it was felt that food intake during this period would introduce an additional variable into both the urinary and fecal excretion.

RESULTS

Growth and survival

Table 1 summarizes the data on growth and survival of deficient rats maintained on the 24% and on the 64% casein diets. Growth and survival on the high protein level were in striking contrast to those on the lower level. Furthermore, the superiority of the high-protein diet increased with age. Both growth and survival on the 64% casein diet were slightly but significantly better by 60 days of age and markedly better by 90 days of age. Few animals maintained on the 24% casein diet survived 120 days (only 21% under the experimental conditions), whereas animals maintained on the 64% casein diet survived indefinitely once they were past the critical age of 50–70 days. Animals have been maintained as long as 12 months on the high protein diet deficient in pantothenic acid, their body weight and general condition improving with each month. It will be noted that the few deaths on the 64% level occurred, with only a single exception, before 60 days.

[5] Mazola.

Apparently 1 or 2 rats in each series cannot become adjusted to the high protein diet in time for the sparing action to be effective.

The typical symptoms of pantothenic acid deficiency observed in the groups receiving the 24% casein level (greying, porphyrin deposition, dermatitis, and hemorrhagic adrenals) were ameliorated at the 64% level. Greying occurred on the

TABLE 1

Effects of dietary protein level on growth and survival of pantothenic acid deficient rats.

% CASEIN	NO. OF RATS	AV. WT. DAY 21	AV. WT. DAY 60	SURVIVAL DAY 60	AV. WT. DAY 90	SURVIVAL DAY 90	AV. WT. DAY 120	SURVIVAL DAY 120
		gm	*gm*	%	*gm*	%	*gm*	%
24	13 ♀	47	84	54	93	8	...	0
	10 ♂	48	110	80	110	50	94	10
	12 ♂	47	100	58	120	42	132	25
	15 ♂	48	94	67	118	40	137	40
	10 ♂	48	102	70	122	10	146	10
	17 ♂	50	111	73	128	53	136	29
Averages	77 ♀ and ♂	48	101	68	120	35	134	21
64 [1]	12 ♀	48	115	83	170	83	202	83
	10 ♂	47	141	90	234	90	300	90
	12 ♂	47	124	92	205	83	255	83
	5 ♂	48	112	100	170	100	200	100
Averages	39 ♀ and ♂	47	124	90	197	87	243	87

[1] The animals maintained on the 64% casein diet are littermates of the corresponding first 4 groups on the 24% casein diet. The additional animals on the 24% level are included to show the variation in growth and survival on this diet.

high-protein level at the same time it was observed on the lower level (45–65 days of age) but in the majority of animals maintained on the high protein level the original fur color began to return between 90 and 120 days of age. Spontaneous cures of greying have been reported for rats receiving suboptimal levels of pantothenic acid by Henderson et al. ('42) and by Unna and Richards ('42). Porphyrin deposition and

dermatitis were not observed at the high protein level; however, hemorrhagic adrenals were found in the few rats that died at an early age.

Urinary and fecal excretion of pantothenic acid

It is evident from the data summarized in figure 1 that the urinary excretion of pantothenic acid was significantly higher for rats on the 64% casein diet than for those on the 24% casein diet. Urinary excretion on the high casein level increased directly with age and paralleled the increased growth and survival observed on this diet. This increasing superiority of the high protein diet with age may be due to the fact that the pantothenic acid requirement of the rat decreases with age (Unna and Richards '42). The daily urinary excretion of pantothenic acid for 56 rats maintained on the 64% casein diet averaged 6.0 μg, beginning with 3.3 μg (day 30–60) and 4.6 μg (day 60–90) up to 6.7 μg (day 90–120) and 7.1 μg (day 120–165). Additional determinations carried out on 21 older rats, 225–285 days of age (not shown on the figure), averaged 10.7 μg daily. In contrast, the daily urinary excretion of pantothenic acid for 45 rats maintained on the 24% casein diet averaged 0.9 μg, with corresponding figures for the same age groups of 0.8 μg, 0.8 μg, 1.1 μg, and 1.1 μg, respectively. The vitamin excretion on this diet was similar but slightly higher (as would be expected) than the values reported by Henderson et al. ('42) for pantothenic acid deficient rats maintained on an 18% casein diet.

The fecal excretion of pantothenic acid varied markedly, regardless of the casein level. Up to 90 days of age, at which time growth and survival on the high protein level are already markedly better than on the lower level, there was no significant difference in fecal excretion for the 2 protein levels.[6]

[6] Recently, Wright and Skeggs ('46) have reported that the fecal excretion of the B vitamins (including pantothenic acid) varied directly with the protein level when diets supplemented with 8 B vitamins but furnishing deficient or suboptimal amounts of casein (5%–18%) were used. It may be noted that the lowest casein level used in the present study was 24%.

After 90 days of age the high protein group tended to excrete more pantothenic acid in their feces. This tendency is shown in the averages for the dietary groups and for the different

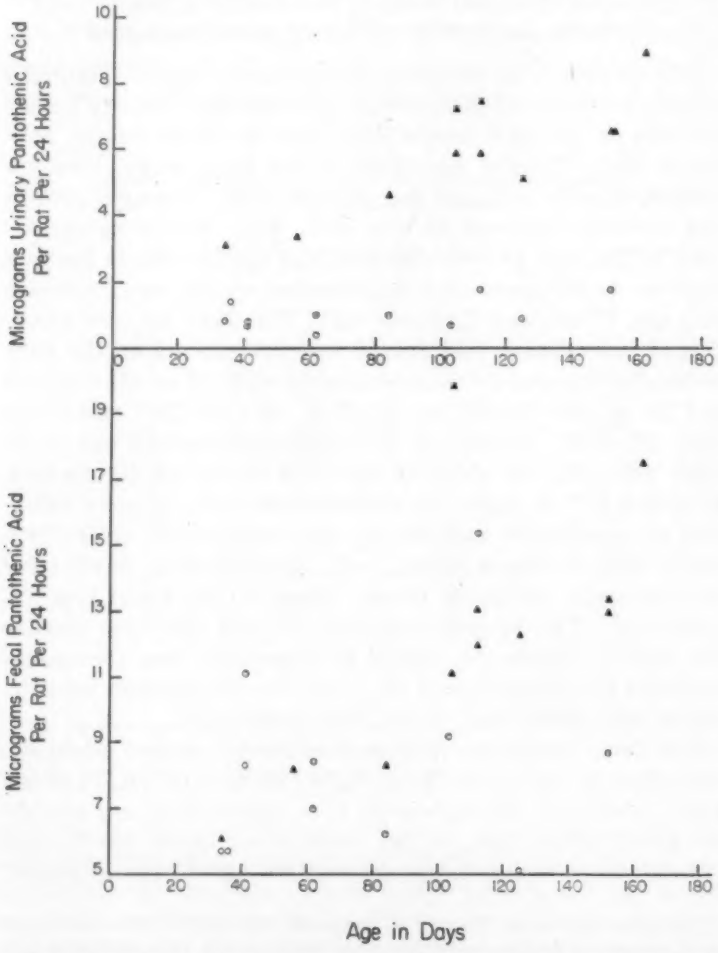
Micrograms Urinary Pantothenic Acid Per Rat Per 24 Hours
Micrograms Fecal Pantothenic Acid Per Rat Per 24 Hours
Age in Days

Fig. 1 Urinary and fecal pantothenic acid excretion by pantothenic acid deficient rats maintained on diets containing 24% and 64% casein. ○ = 24% casein diet, ▲ = 64% casein diet.

age groups on the diet. Fifty-eight rats maintained on the 64% casein diet averaged 12.4 μg fecal pantothenic acid per 24 hours, beginning with 7.2 μg (day 30–60) and 8.3 μg (day 60–90) to 14.1 μg (day 90–120) and 14.2 μg (day 125–165). In addition, determinations carried out with 23 older animals, 225–285 days of age (not shown on the figure) averaged 20.9 μg daily. On the 24% casein diet 45 rats averaged 8.0 μg fecal pantothenic acid per 24 hours and the corresponding figures for the same age groups were 7.8 μg, 7.5 μg, 11.5 μg, and 6.5 μg, respectively. Few determinations were carried out on rats maintained on the 24% casein level over 120 days of age because of the small number of animals surviving this period.

Mention has been made of a possible effect of food intake on fecal excretion. Fecal excretion of pantothenic acid was measured on a group of 9 rats maintained on the 64% casein diet and 160–165 days of age. When fasted the fecal pantothenic acid per 24 hours averaged 17.5 μg (range 9–24). When given food the fecal pantothenic acid over a period of 5 days averaged 28.2 μg (range 15–48). The averages for successive 24- or 48-hour collection periods were 28.2 μg (range 17–34), 32.9 μg (range 25–39), and 25.9 μg (range 15–48). The variation in fecal excretion for individual rats during these periods were as follows: 15–26, 17–28, 18–25, 18–33, 19–36, 24–48, 25–34, 25–38, and 27–39 or a difference of 7–24 μg between the minimum and maximum values for any individual rat. These figures are given to indicate the extreme variability in fecal excretion, especially when the animals are given food. This marked variability is one reason for pooling fecal samples for dietary groups.

The data given on fecal excretion of pantothenic acid at the 2 protein levels do not indicate that increased intestinal synthesis of the vitamin occurred at the high protein level during the first 90 days of age. Although the fecal excretion at the high protein level increased with age, this may be due to the increase in body weight and correspondingly in size of cecum (Taylor et al., '42) rather than to the protein level

per se. The data given on urinary excretion clearly show an increase in vitamin excretion at the high protein level. There is, obviously, no relative decrease in the pantothenic acid requirement because of a decreased vitamin loss by urinary excretion on this diet.

Effect of intermediate protein levels

The marked differences in growth, survival and urinary excretion at the 2 casein levels, 24% and 64%, suggested the desirability of testing the intermediate levels of casein. Twelve litters of 21-day-old male rats were divided into equivalent groups and placed on the deficient diets containing 24%, 34%, 44%, 54%, and 64% casein, with corresponding decreases in the percentage of sucrose. The rats were observed until 150 days of age. Urinary and fecal pantothenic acid were measured during the latter half of the experimental period, i.e., the ninth, twelfth, thirteenth and nineteenth weeks of the experimental period.

Growth (fig. 2), survival and urinary pantothenic acid (table 2) were proportional to the protein level. In agreement with the data on fecal excretion for the 24% and 64% casein diets, there was no correlation between fecal pantothenic acid and the protein level. The sparing action of high casein diets, as judged by growth, survival and urinary excretion of pantothenic acid, was directly proportional to the casein level of the deficient diets.

Analysis of casein

The marked increase in urinary excretion of pantothenic acid by rats maintained on the higher protein levels prompted a reexamination of the pantothenic acid content of the casein used in the purified diets and of other types of purified caseins. Microbiological assays using water extraction (and heat sterilization) had previously indicated that 3 types of casein prepared in the laboratory, namely acid-washed, ether-extracted, and alcohol-extracted caseins, contained insignificant

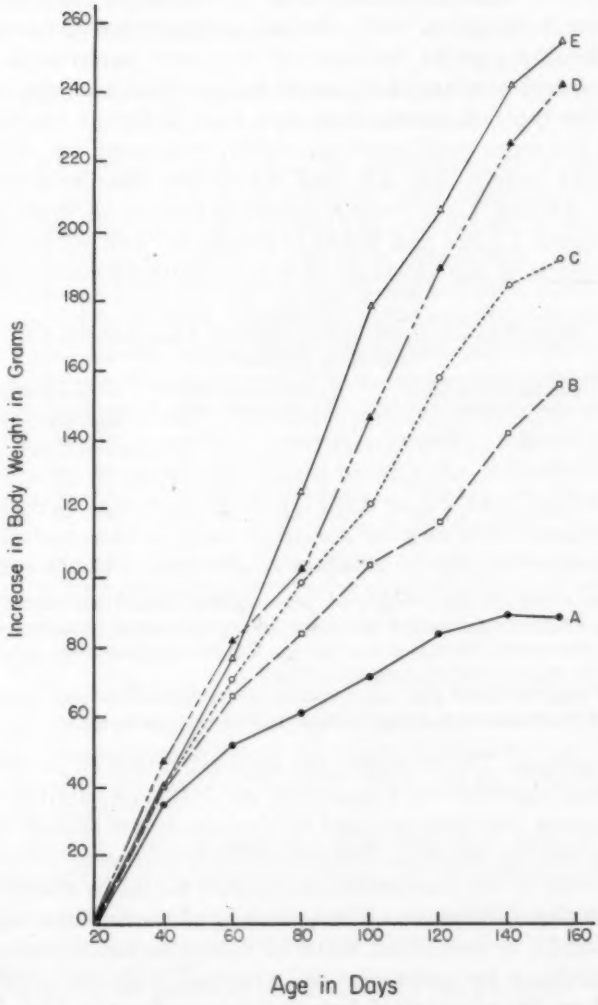

Fig. 2 Growth of pantothenic acid deficient male rats maintained on diets varying in casein level. A = 24% casein diet, B = 34% casein diet, C = 44% casein diet, D = 54% casein diet, E = 64% casein diet.

amounts of pantothenic acid (0.1 to 0.3 μg per gm casein). Enzymatic digestion with clarase revealed the presence of considerably greater amounts of "bound" pantothenic acid in all samples of purified casein tested. The amount varied with the type of purification and with different batches in which the same purification procedure was used, i.e., alcohol-extracted casein, 2.8, 1.8, and 1.5 μg per gm; acid-washed casein 2.8 and 1.9 μg; ether-extracted casein 2.0 and 1.8 μg; GBI casein 1.3 μg; and SMACO casein 0.9 and 0.7 μg. Each value given is the average of 2 to 3 determinations on the

TABLE 2

Effect of dietary protein level on pantothenic acid excretion and survival of deficient rats.

% CASEIN	URINARY PANTOTHENIC ACID μG/RAT/24 HRS.[1]		FECAL PANTOTHENIC ACID μG/RAT/24 HRS.[1]		% SURVIVAL			
	Average	Range	Average	Range	Day 60	Day 90	Day 120	Day 150
24	1.6 (6) [2]	1.1–1.8	11.4 (7)	4.0–15.4	58	42	25	17
34	1.8 (16)	1.6–2.0	12.4 (15)	8.1–19.3	75	50	50	50
44	3.5 (13)	2.7–3.7	9.1 (14)	6.7–15.5	87	67	50	50
54	5.8 (13)	2.4–6.2	12.9 (17)	4.0–15.5	100	75	67	67
64	6.6 (25)	3.0–7.5	12.2 (26)	9.0–13.2	92	83	83	83

[1] These values are the averages of determinations carried out on the ninth, twelfth, thirteenth and nineteenth weeks of the experiment. Twenty-four-hour samples were pooled for each dietary group. Numbers in parentheses refer to the number of rats on which the average is based.

[2] This value is higher than usual because the determinations were carried out on the few rats surviving during the later half of the experiment.

same sample. These values are approximately twice as high as those reported by Cannon et al. ('45) on similar purified caseins (the enzyme used by Cannon is not specified) but the variability between different batches of casein and slight variations in the purification procedure would be sufficient to explain the differences. The presence of pantothenic acid in a "bound" or combined state in many biological materials and methods for releasing the vitamin from the combined state have been reported by several investigators (Pennington, Snell and Williams, '40; Waisman et al., '42; Wright, '43). It may be mentioned that enzymatic digestion of urine and feces did not increase the pantothenic acid values so that

presumably all pantothenic acid excreted by the rat is in a "free" or uncombined state.

Effect of 24% casein diets supplemented with low levels of pantothenic acid

Since a purified casein [7] free of pantothenic acid was not available, low levels of pantothenic acid equivalent to or greater than the amount contained in the casein of the high protein diets, were added to the 24% casein diet. All diets were prepared from 1 batch of casein and 4 different analyses (with and without ether extraction) were carried out on each diet. Three casein levels (24%, 44% and 64%) were used and in addition 2 supplemented diets containing 24% casein. One supplemented diet contained slightly more pantothenic acid than either of the 2 higher protein diets; the other contained twice as much pantothenic acid as the 44% casein diet and slightly less than twice as much as the 64% casein diet. The diets together with their pantothenic acid content are as follows: 24% casein diet containing 0.3 μg pantothenic acid per gm diet; 2 supplemented diets, 24% casein, with 0.9 and 1.4 μg, respectively; 44% casein diet, 0.7 μg; and 64% casein diet, 0.8 μg.

Five litters of 21-day-old male rats were used and observed until 120 days of age. Urinary and fecal excretion of pantothenic acid was measured at intervals throughout the entire experimental period, i.e., second, fifth, ninth, twelfth and fifteenth weeks.

The data in table 3 show, as expected, that growth [8] and urinary excretion of pantothenic acid by rats maintained on

[7] Recent studies have indicated the possibility of freeing beef blood fibrin from traces of pantothenic acid by washing with water or dilute acid. Further experiments are in progress.

[8] The groups in this experiment were too small (5 rats per group) to obtain much significant data on survival. It may be noted that all other experimental groups reported have contained 10–15 rats per group. 100% of the animals survived 90 days on the 64% casein diet and on the corresponding 24% casein supplemented diet (0.9 μg per gm diet); 60% of the animals survived the same period in the remainder of the groups.

the 24% casein diets with or without supplements were proportional to the pantothenic acid content of the diet. This is in agreement with the studies of Henderson et al. ('42). However, growth and urinary pantothenic acid of littermates maintained on the higher protein levels (44%[9] and 64%)

TABLE 3

Effect of dietary protein level and low levels of pantothenic acid on vitamin excretion and growth of deficient rats.

EXPTL. GROUP		URINARY PANTOTHENIC ACID μG/RAT/24 HRS.[1]		FECAL PANTOTHENIC ACID μG/RAT/24 HRS.[1]		AV. BODY WT.—GM		
Casein %	Pantothenic acid content μg/gm diet	Average	Range	Average	Range	Day 60	Day 90	Day 120
24	0.3	0.7 (14)	0.6–0.9	4.4 (14)	3.2–5.7	79	94	98
24	0.9	1.6 (19)	1.1–2.1	6.8 (19)	5.7–8.4	91	135	163
24	1.4	1.9 (18)	0.8–3.9	5.6 (18)	3.8–10.9	95	144	191
44	0.7	2.2 (13)	1.6–2.8	7.3 (13)	3.9–10.4	107	158	185
64	0.8	4.5 (25)	3.1–5.9	10.9 (25)	6.1–19.9	112	170	200

[1] Values are averages for determinations carried out on the second, fifth, ninth, twelfth and fifteenth weeks of the experiment. Twenty-four-hour samples were pooled for each dietary group. Numbers in parentheses refer to the number of rats on which the average is based.

were markedly greater than on the 24% casein diet with the corresponding pantothenic acid content (0.9 μg per gm diet). When the pantothenic acid content of the 24% casein diet was double or practically double that of the higher protein levels, growth and urinary pantothenic acid were approximately equal to that produced by the 44% casein diet but were still not equivalent to the results obtained with the 64% casein diet. Therefore, it was concluded that the pantothenic acid content of the casein used in the purified deficient diets does not account for all of the sparing action of the

[9] It may be mentioned that every weekly determination of urinary pantothenic acid for rats maintained on the 44% casein diet was higher than the value obtained from the corresponding 24% casein supplemented diet (0.9 μg per gm diet).

high casein diets, although it is responsible for part of the protective action observed.

In regard to fecal excretion of pantothenic acid, the averages showed an apparent increase proportional to the casein level. This was not considered significant in view of the marked variability in fecal excretion (see fig. 1). The tendency of the fecal excretion to increase with age on the high protein levels may or may not result in an increased average value (compare tables 2 and 3).

DISCUSSION

The data reported in this paper indicate that neither increased intestinal synthesis as measured by fecal excretion nor a decreased vitamin loss by urinary excretion are responsible for the sparing action of high casein diets on the pantothenic acid requirement of the rat. Furthermore, the pantothenic acid content of the casein used in the purified diet, while exercising an effect, cannot account for the protective action of such diets. Therefore, it would appear that there is a real decrease in the pantothenic acid requirement of the rat when higher protein intake occurs.

It is, of course, possible that some component of casein may replace or be converted to pantothenic acid in the body in a manner similar to the tryptophane-niacin interrelationship (Krehl et al., '46). This would explain the increased urinary excretion of pantothenic acid on the high casein diets. It is also possible that this increased urinary pantothenic acid excretion may be merely a reflection of the increased food intake on such diets, an increase caused by some factor(s) concerned in the efficient utilization of pantothenic acid. Such a factor might be a known or unknown component of casein as previously suggested (Nelson and Evans, '45), e.g., amino acids, phosphorus, or unknown factors similar in their action to biotin and folic acid which are reported (Wright and Welch, '43, '44) to be necessary for pantothenic acid utilization. It has also been suggested recently (Wright and Welch, '46)

that the necessity of a protein substrate for pantothenic acid combination in blood and tissues may explain the sparing action of high protein diets. On the other hand, the observed sparing action may actually be due to the decreased level of carbohydrate metabolism. Further investigations of these possibilities are in progress.

SUMMARY

Studies of urinary and fecal excretion of pantothenic acid by deficient rats maintained on purified diets differing in casein level gave the following results:

1. Urinary pantothenic acid excretion was significantly higher on diets containing 64% casein than on diets containing 24% casein and paralleled the increased growth and survival observed at the higher protein level. Urinary excretion increased with age on the 64% casein diet.

2. Fecal pantothenic acid excretion was higher when rats were fed than when they were fasted and was notable for its variability. During the first 90 days there was no significant difference in fecal excretion, regardless of the casein level. With increasing age and body weight there was a tendency for fecal output to increase on the higher protein level.

3. When intermediate levels of casein were used, growth, survival and urinary pantothenic acid (but not fecal pantothenic acid) were proportional to the casein level.

4. When low levels of pantothenic acid equivalent to that present in the high casein diets (by microbiological assay) were added to the 24% casein diet, growth and urinary pantothenic acid were not equal to those observed on the higher casein diets. When approximately double the equivalent amount of pantothenic acid was added to the 24% casein diets, the results were equivalent to those observed at the 44% casein level but were still not equal to those at the 64% casein level.

Neither increased intestinal synthesis of pantothenic acid as measured by fecal excretion nor a decreased vitamin loss by urinary excretion are responsible for the sparing action

of high casein diets on the pantothenic acid requirement of the rat. The pantothenic acid content of the casein used in the purified diet while exercising an effect can not fully account for the protective action of such diets.

LITERATURE CITED

CANNON, M. D., R. K. BOUTWELL AND C. A. ELVEHJEM 1945 The vitamin content of casein. Science, *102:* 528.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 460.

HENDERSON, L. M., J. M. MCINTIRE, H. A. WAISMAN AND C. A. ELVEHJEM 1942 Pantothenic acid in the nutrition of the rat. J. Nutrition, *23:* 47.

KREHL, W. A., J. DE LA HUERGA AND C. A. ELVEHJEM 1946 Tryptophane studies. I. The effect of niacin on the utilization of tryptophane. J. Biol. Chem., *164:* 551.

NEAL, A. L., AND F. M. STRONG 1943 Microbiological assay for pantothenic acid. J. Ind. and Eng. Chem., Anal. Ed., *15:* 654.

NELSON, M. M., AND H. M. EVANS 1945 Sparing action of protein on the pantothenic acid requirement of the rat. Proc. Soc. Exp. Biol. Med., *60:* 319.

PENNINGTON, D., E. E. SNELL AND R. J. WILLIAMS 1940 An assay method for pantothenic acid. J. Biol. Chem., *135:* 213.

STRONG, F. M. 1944 Microbiological assay methods for riboflavin, niacin, and pantothenic acid. (Mimeographed, Dept. of Biochemistry, University of Wisconsin.)

STRONG, F. M., R. E. FEENEY AND A. EARLE 1941 Microbiological assay for pantothenic acid. J. Ind. and Eng. Chem., Anal. Ed., *13:* 566.

TAYLOR, A., D. PENNINGTON AND J. THACKER 1942 The vitamin requirements of cecectomized rats. Univ. of Texas Publication no. 4237, p. 72.

UNNA, K., AND G. V. RICHARDS 1942 Relationship between pantothenic acid requirement and age in the rat. J. Nutrition, *23:* 545.

WAISMAN, H. A., L. M. HENDERSON, J. M. MCINTIRE AND C. A. ELVEHJEM 1942 The effect of enzymatic digestion on the pantothenic acid content of meats determined by the microbiological method. J. Nutrition, *23:* 239.

WRIGHT, L. D. 1943 The state of pantothenic acid in blood. J. Biol. Chem., *147:* 261.

WRIGHT, L. D., AND H. R. SKEGGS 1946 Vitamin B complex studies with diets differing in the level of protein. Proc. Soc. Exp. Biol. Med., *63:* 327.

WRIGHT, L. D., AND A. D. WELCH 1944 Folic acid, biotin and pantothenic acid deficiency and the liver storage of various vitamins in rats fed succinylsulfathiazole in highly purified rations. J. Nutrition, *27:* 55.

———— 1943 The role of "folic acid" and biotin in the utilization of pantothenic acid by the rat. Science, *97:* 426.

THE EFFECT OF MIXED TOCOPHEROLS ON THE UTILIZATION OF VITAMIN A IN THE RAT [1]

JANET M. LEMLEY, R. A. BROWN, O. D. BIRD AND A. D. EMMETT

Research Laboratories, Parke, Davis and Co., Detroit, Michigan

(Received for publication March 6, 1947)

The relation of tocopherols or vitamin E to vitamin A utilization was first reported by Moore ('40) after he observed that a lack of adequate vitamin E in the diet of rats receiving vitamin A supplements was associated with low reserves of vitamin A in the liver. Results of studies carried out the following year in cooperation with Davies (Davies and Moore, '41) showed that in the livers of rats maintained on a diet deficient in both vitamins A and E, the vitamin A store disappeared much more rapidly than did that from livers of animals receiving dl-α-tocopherols. It was pointed out by these authors that the tocopherols had been used in vitro for stabilizing solutions of vitamin A in fats and that these substances might possibly exert a similar effect in vivo. The inter-relationship of vitamins A and E was subsequently studied by Bacharach ('40), Sherman ('41), Quackenbush et al. ('42), Hickman et al. ('42, '44), Guggenheim ('44), Harris et al. ('44), Popper and Volk ('44), Gridgeman ('44), Sanders et al. ('44), Dattatreya Rao ('45) and Kemmerer et al. ('47).

The object of the work presented in this paper was to investigate further the covitamin activity of tocopherols as it

[1] This material was presented at the American Chemical Society Meeting, September, 1946, at Chicago, Ill.

is reflected in increased growth and in the storage of vitamin A in the livers of rats. These experiments have been designed to test previous findings reported concerning vitamins A–E synergy and to gain additional information regarding the mode of action of vitamin E. More specifically the objectives of this study are: (1) to determine the effect of tocopherols on the growth response to vitamin A; (2) to investigate the influence of the tocopherol content of the diet on the growth obtained with the U.S.P. bioassay procedure for vitamin A; (3) to determine whether increasing amounts of vitamin A will compensate for a lack of vitamin E; (4) to observe the covitamin activity of tocopherols administered with vitamin A at different intervals of time and by various methods; (5) to investigate the effect of tocopherols on the storage of vitamin A in the liver of the rat when given in combination with vitamin A to animals subsisting on a vitamin A–E deficient diet.

PROCEDURE

The general procedure involved in these experiments is outlined below and all deviations from this procedure will be discussed in detail in separate sections under "Results."

In order to observe the effect of tocopherols on vitamin A it was first necessary to maintain the experimental animals on diets deficient in both vitamins until the animals showed signs of vitamin A deficiency as manifested by declining weight. The standard U.S.P. diet for vitamin A bioassay was used with the following modifications: (1) Jones and Foster ('42) salt mixture was substituted for that described in the Pharmacopoeia; (2) 6 U.S.P. units of vitamin D (irradiated ergosterol) per gram of ration were added and (3) the yeast and starch were washed with acetone. The type of vegetable oil was varied; diet 1 contained olive oil, diet 2 cottonseed oil and diet 3 corn oil. The tocopherols were eliminated from the diet by substituting olive oil for cottonseed or corn oil. To be certain that the ration containing olive oil was deficient in vitamin E, groups of males and females were raised to

maturity on this diet and mated. No litters were born, whereas, a comparable group of rats raised on a similar ration, supplemented with 0.3 mg daily of mixed tocopherols cast and reared normal litters. The same lot of olive oil was used throughout the experiment.

Weanling rats, distributed approximately in equal numbers according to sex (unless otherwise stated), age, and weight, were maintained on diet 1 in air conditioned quarters (75°F., 40% relative humidity). When their weights remained stationary or declined for a period of 7 successive days the rats were considered to be depleted of their vitamin A reserves. This required from 25 to 35 days. The rats were then divided into experimental groups and treated with various supplements for a 28-day test period. The oil diluent employed in administering the vitamin A and mixed tocopherols was the same as that used in the ration; either olive, cottonseed or corn oil. The supplements were made fresh each week and stored under CO_2 in the refrigerator when not in use.

It has been reported that the vitamin E content of oils varies widely (Sherman, '41; Dattatreyo Rao, '45) and in consequence an effort was made to ascertain the effect of this variation on growth. Whenever a vitamin A supplement was used, it was either the U.S.P. Reference Oil II or a vitamin A distillate [2] (no. 78794) diluted in olive oil. The vitamin E supplement was a mixture of natural tocopherols.[2] With 2 exceptions all vitamin E supplements were given in 1 of the vegetable oils and administered orally. In 1 experiment described in Section I, the tocopherols were first dissolved in cottonseed oil and then incorporated in the diet. In another experiment where the vitamin was administered by injection (Section III) the tocopherols were solubilized with "Tween 20" [3] and given in an aqueous solution.

[2] Obtained from Distillation Products, Rochester, N. Y.

[3] Obtained from Atlas Powder Company, Wilmington, Delaware. Twelve gm of "Tween 20" were mixed with 970 mg of a 31% solution of natural mixed tocopherols concentrate and made to a volume of 100 ml with distilled water.

RESULTS

I. The supplementary action of tocopherols when administered to vitamin A depleted rats receiving the U.S.P. Reference cod liver oil

The animals receiving diet I (table 1) without the addition of tocopherols, made an average increase in weight of 16 gm as compared with the 29 gm for a comparable group receiving the tocopherol supplement. This indicates that under the conditions of the experiment, tocopherols were necessary for optimum utilization of vitamin A. It was also noted that variable responses were obtained when the cottonseed oil diet (diet II) was used. The data show that the supplementing

TABLE 1

The supplementary effect of tocopherols added to olive, cottonseed and corn oils on the growth response of vitamin A depleted rats receiving U.S.P. Reference cod liver oil.

GROUP NO.	NO. RATS	DIET NO.	TYPE OF VEGETABLE OIL	TOCOPHEROL SUPPLEMENT [1]	REFERENCE COD LIVER OIL	WEIGHT GAIN IN 28 DAYS
				mg/rat/day	*units/day*	*gm*
1	12	I	Olive oil	....	2.04	16 ± 3.9 [3]
2	14	I	Olive oil	0.025	2.04	29 ± 4.0
3	17	II	Cottonseed oil lot I [2]	....	2.04	34 ± 5.5
4	13	II	Cottonseed oil lot I [2]	0.025	2.04	35 ± 3.9
5	22	II	Cottonseed oil lot II [2]	....	2.04	34 ± 3.2
6	16	II	Cottonseed oil lot II [2]	0.3 (per 10 grams ration)	2.04	46 ± 3.6
7	21	III	Corn oil	....	2.04	41 ± 3.4
8	20	III	Corn oil	0.3	2.04	44 ± 3.3

[1] Administered in terms of mixed tocopherol content.
[2] Two different lots of cottonseed oil were used in these studies.
[3] Standard error.

of diet II with 0.025 mg per day of tocopherols failed to increase the growth promoted by the vitamin A supplement although the addition of 0.3 mg of the vitamin per 10 gm of a similar ration containing another lot (lot II) of cottonseed oil did measurably increase the weight response. From the similarity in growth responses of the animals receiving the ration unsupplemented with tocopherol in which 2 different lots of cottonseed oil were used (groups 3 and 5) it seems probable that the 2 samples of oil contained equal amounts of tocopherols, although the amount available was inadequate. If the data at hand justify a comparison of the 2 lots of oil, it may be postulated that the 0.025 mg supplement of tocopherol was insufficient and that 0.3 mg were required for a further increase in growth. It should be pointed out that tocopherols incorporated in the ration (group 6) measurably increased the response to vitamin A although Hickman and associates ('42, '44) have stated that the synergistic effect of vitamin E is more significant when fed separately.

When the corn oil diet was used, the addition of tocopherols had little effect on the response to vitamin A indicating that the corn oil contained an adequate amount of tocopherols.

These data show that there is sufficient variation in the tocopherol content of different types of vegetable oils to affect the rate of growth of test animals during the usual vitamin A assay and therefore this factor should be considered in the standard U.S.P. vitamin A assay. The data confirm recent evidence that dietary supplementation with an adequate amount of tocopherol is necessary in order to secure maximum weight response to a given dose of vitamin A (Hickman et al., '44; Sanders et al., '44; Dattatreya Rao, '45).

II. The effect of tocopherols on the growth response of vitamin A depleted rats when given increasing amounts of vitamin A

Prior to the 28-day treatment period all rats were maintained on diet I containing olive oil until depleted of vitamin A. The diet was unchanged during the subsequent growth test

except in the case of animals receiving daily oral supplements as indicated in table 2. One series of test animals was fed tocopherols orally at a level of 0.3 mg per day, the supplement being in combination with increasing amounts of vitamin A. The rats of the second series received comparable amounts of the vitamin A supplement without added tocopherol. The data show that the presence of tocopherols increased the growth response of rats to vitamin A within certain limits. These data also show that as the vitamin A intake was raised above 4.08 units per day, the covitamin E activity was no longer

TABLE 2

The effect of tocopherols on the growth response of vitamin A depleted rats, when given increasing amounts of vitamin A.

SUPPLEMENT VITAMIN A	WITH TOCOPHEROLS 0.3 MG/DAY/RAT		WITHOUT TOCOPHEROLS	
	No. rats	Weight response in 28 days	No. rats	Weight response in 28 days
units/day [1]		*gm*		*gm*
0	11	−9	17	−16
2.04	19	+ 43 ± 3.1 [2]	17	+ 32 ± 5.6 [2]
4.08	20	+ 66 ± 1.6	21	+ 54 ± 2.5
8.16	20	+ 79 ± 5.9	21	+ 84 ± 4.5
16.32	20	+ 86 ± 4.1	21	+ 87 ± 5.8
32.64	19	+ 94 ± 6.0	22	+ 97 ± 7.0

[1] The supplements were diluted in olive oil.

[2] Standard error.

apparent. If it is assumed that tocopherols have a similar effect on carotene and vitamin A, then these data indirectly support the statement of Harris, Kaley and Hickman ('44) that "small quantities of tocopherol are synergistic with small and moderate intakes of carotene. . . . There is a definite ratio between carotene and tocopherol intakes; so that the potency of larger quantities of carotene benefits from increased quantities of tocopherol." In order to show optimum utilization of large amounts of vitamin A it may be necessary to raise the level of vitamin E or use some criterion other than growth.

III. The effect of tocopherols and vitamin A administered at various time intervals to vitamin A depleted rats

A state of vitamin A depletion was produced in all animals by feeding the U.S.P. diet containing olive oil (diet I). The test animals were then divided into experimental groups and treated for a 28-day period with equal doses of tocopherols and similar levels of vitamin A administered as indicated in

TABLE 3

The synergistic effect of tocopherols and vitamin A administered at various time intervals to vitamin A depleted rats.

GROUP NO.	NO. RATS	TIMES TREATED PER WEEK	SUPPLEMENTS [1]		WEIGHT GAIN OR LOSS IN 28 DAYS
			Tocopherols	Vitamin A	
					gm
1	35	6	None	Daily — Oral	+ 21 ± 3.0 [2]
2	20	6	Daily Oral	None	— 8.5
3	37	6	Daily Oral	Daily — Oral	+ 48 ± 2.8
4	18	6	Daily Oral A.M.	Daily — Oral P.M.	+ 43 ± 3.1
5	18	3	Mon., Wed., Fri., Oral	Tues., Thurs., Sat., Oral	+ 39 ± 3.3
6	19	2	Wed., Sat., Oral	Wed., Sat., Oral	+ 37 ± 4.5
7	19	2	Wed., Sat., Oral	Mon., Thurs., Oral	+ 37 ± 3.7
8	57	6	Daily — Injected (Solubilized tocopherol used)	Daily — Oral	+ 40 ± 2.4

[1] Supplements — tocopherols — 1.8 mg per week; vitamin A — 12.24 units per week. This was equivalent to 0.3 mg tocopherols daily and 2.04 units of vitamin A daily.

[2] Standard error.

table 3. With the exception of the aqueous tocopherol used for injection (group 8) all supplements were diluted in olive oil and given orally.

Two control groups (1 and 2) were included, the animals of 1 group receiving vitamin A only, while those of the other group received the tocopherols alone. It may be observed that vitamin A promoted some growth in the test animals while the animals which were given tocopherols following complete deprivation of vitamin A lost weight. The animals of group 3

which served as a positive control or as a standard of comparison received orally each day both vitamin supplements dissolved together in olive oil. As would be expected these animals responded with a maximum gain in weight.

The mode of treatment was modified for the animals of groups 4, 5, 6 and 7 in that although they received the same amount of vitamins A and E per week, the interval of administration was varied. From the results obtained it appears that tocopherols given separately exerted slightly less synergistic activity than when given in combination with vitamin A. It should be pointed out, however, that the difference involved was too slight to be truly significant. Hickman et al. ('42, '44) reported that the synergistic effect of vitamin A and E was definitely reduced when they were fed on alternate days rather than simultaneously but the observations of these authors were made while using somewhat smaller doses of the vitamins.

Daily parenteral injection of an aqueous solution of tocopherols with oral administration of vitamin A was employed with animals of group 8. The average weight responses of the animals receiving this treatment were slightly lower than optimum but not significantly different from the average gain of the groups given both supplements orally.

The conditions of these experiments have been designed to gain additional information concerning the site and mechanism of covitamin E activity. Both Quackenbush et al. ('42) and Hickman et al. ('44) have postulated that the action of tocopherol is a chemical one probably occurring in the gastrointestinal tract. Hickman based his conclusion on the reduced synergism he observed when the vitamin and covitamin are given at different times or by different routes. The present data fail to corroborate these observations, however, and the response noted when tocopherol was injected parenterally suggests that its supplementary action on vitamin A is not confined to the digestive tract. A publication by Popper and Volk ('44), in which it was reported that fluorescent microscopic examination of the rat intestine showed no increased

absorption of vitamin A after the administration of tocopherols, lends indirect support to our findings. It appears that the covitamin activity of tocopherol is more extensive in scope than has been generally assumed and is not a simple chemical reaction limited to the digestive tract.

IV. The effect of tocopherols on the storage of vitamin A in the liver of the rat

With the exception of 2 groups of male rats (groups 15 and 16) which had been included for the purpose of comparison, females were used exclusively for the studies on liver storage of vitamin A. All experimental animals were provided with diet I, the U.S.P. vitamin A-free diet containing olive oil, until depleted of vitamin A. The test animals were then divided into groups and the animals of the respective groups were treated as indicated in table 4. In this treatment the same diet was continued but a daily supplement of vitamin A distillate in olive oil alone or in combination with tocopherols in olive oil was given orally. The daily allowance of tocopherols was maintained at 0.3 mg but the level of vitamin A ranged from 63 to 4,000 U.S.P. units. The animals comprising groups 1–12 were treated for 3 days with daily supplements of the vitamin A distillate administered alone to 1 group and in combination with tocopherols to a comparable group, the vitamin A supplement being increased with each succeeding pair of animal groups. After 3 days of vitamin supplementation the animals were rested for 1 day and on the fifth day were killed by decapitation. The livers from each group of rats were weighed, pooled, homogenized in a Waring blendor and samples taken for the determination of vitamin A. The method used for the estimation of vitamin A in liver tissue has been outlined in a previous publication (Lemley, Brown, Bird and Emmett, '47).

The results presented in table 4 indicate that the effect of tocopherols on the storage of vitamin A in the liver of the rat cannot be demonstrated when a short term supplementing period is employed. In some instances the animals receiving

tocopherols had slightly larger liver stores of vitamin A than those treated with vitamin A alone. For example, those animals which had received vitamin A supplements of 4,000 units daily stored 4,150 units of vitamin A, while those receiving tocopherols in addition to this vitamin A supplement were found to have an average of 4,480 units in the liver. These

TABLE 4

The effect of tocopherol intake on the storage of vitamin A in the liver of the rat.

GROUP NO.	NO. RATS	SEX	TREATMENT			LIVER STORAGE OF VIT. A	% VIT. A STORED
			Duration of treatment	Tocopherol supplement	Vitamin A supplement	I.U. of A/ Total liver	
				mg/day	*units/day*		
1	4	F	Three days	0.0	63	0	0
2	5	F	Three days	0.3	63	0	0
3	6	F	Three days	0.0	250	301	40.0
4	6	F	Three days	0.3	250	245	32.7
5	7	F	Three days	0.0	500	525	35.0
6	7	F	Three days	0.3	500	520	34.8
7	8	F	Three days	0.0	1000	960	32.7
8	5	F	Three days	0.3	1000	1032	34.4
9	8	F	Three days	0.0	2000	1835	30.6
10	5	F	Three days	0.3	2000	2360	39.4
11	8	F	Three days	0.0	4000	4150	34.6
12	3	F	Three days	0.3	4000	4480	37.3
13	8	F	Three months	0.0	100	718	10.0
14	8	F	Three months	0.3	100	1228	17.0
15	4	M	Three months	0.0	100	495	6.9
16	4	M	Three months	0.3	100	1360	18.9
17	4	F	Six months	0.0	100	2270	15.8
18	10	F	Six months	0.3	100	3400	23.6
19	11	F	Six months	0.0	200	6988	24.3
20	10	F	Six months	0.3	200	9025	31.3

differences are not considered significant, however, since it has been reported that large variations in liver reserves of vitamin A are found in similar animals receiving identical treatments (Bacharach, '40).

It should be pointed out that in these studies large doses of vitamin A were employed in order to produce appreciable liver storage in a short time and that any increase in the

liver content of vitamin A due to the addition of tocopherols would be relatively slight. The data in table 4 indicate that there is no marked benefit resulting from taking vitamin A and E together at doses sufficient to cause vitamin A liver storage although there does appear to be a trend toward higher liver stores when tocopherols are given with daily doses of 1,000 to 4,000 units per day of vitamin A. But, as has been mentioned above, these differences are not significant.

Guggenheim ('44) has recently reported that the daily administration of vitamin E in combination with 100 units of vitamin A on 2 successive days increased the liver storage of vitamin A. After the vitamin A was given alone he found 18.5 units per gm of liver tissue but when the same amount of vitamin A was fed with 0.1 to 10 mg of tocopherols, the liver reserves of vitamin A ranged from 22 to 45 units per gm of tissue. In the experiments of this author lower doses of vitamin A were used and the effect of tocopherols became apparent, although our results indicated that tocopherols given with high doses of vitamin A had no noticeable effect on liver storage.

In the later experiments a longer treating period, either 3 or 6 months, was adopted. After the animals had been depleted of vitamin A they were maintained on diet I supplemented as indicated in table 4. In order to be certain that vitamin E was not present in the basal diet, breeding records were also kept on representative females from groups 13, 14, 15 and 16. Six of the females in group 13 which were being fed an olive oil diet unsupplemented with tocopherols became pregnant but no litters were cast, thus indicating that resorption had occurred owing to a lack of vitamin E. Six of the 8 females in group 14 receiving daily supplements of tocopherols bore normal litters. The breeding records of the animals in groups 13 and 15 definitely established the deficiency of vitamin E in the diet. After the rats had undergone treatment for 3 months they were killed and the livers were assayed for vitamin A, the livers from the 2 groups of males

(groups 15 and 16) being similarly assayed. It was thought that pregnancy and lactation might affect the vitamin A storage ability of the females but when their liver reserves were determined and compared with those of males, similar vitamin values were noted. In consequence it was assumed that although these animals had previously been used for breeding experiments the storage of vitamin A in their livers was a reliable indication of the effect of tocopherol treatment. From the results reported in table 4 it can be seen that the females depleted of tocopherols (group 13) stored an average of 718 units of vitamin A while the females of group 14, which had received the tocopherols, stored 1,228 units and likewise the addition of tocopherols to the diet of male rats raised the liver content of vitamin A from 495 to 1,360 units. It was, therefore, concluded that the daily administration of 0.3 mg of tocopherols over an extended period (3 months) increased the liver storage resulting from a daily intake of 100 units of vitamin A.

The storage of vitamin A in the livers of the rats was also determined after feeding a daily supplement of 100 or 200 units of vitamin A alone or combined with 0.3 mg of tocopherols for a period of 6 months. The effect of tocopherols feeding on the storage of vitamin A is evident when one compares the average liver storage of 2,270 units as reported for the animals of group 17 on vitamin E deficient diet with a storage of 3,400 units as found for the animals of group 18 which had received the tocopherol supplement. Similar results were obtained when the vitamin A intake was raised to 200 units daily. The data presented in table 4 show that animals treated for 6 months stored approximately 3 times as much vitamin A in their livers as did similar animals which had been treated for only 3 months. This increase occurred irrespective of tocopherol supplementation and the effect of tocopherols on the liver storage was somewhat less significant at the end of the 6-month feeding period than it had been at the end of the 3-month period. It may also be noted that the increase in liver storage promoted by the inclusion of

tocopherols was less when a daily level of 200 units of vitamin A was given than when the previously mentioned 100-unit daily supplement was employed. In other words, in these experiments the favorable effect of tocopherols on the storage of vitamin A in the liver of the rat was diminished by extending the supplementing period and by raising the daily intake of vitamin A. It is probable that the covitamin activity of tocopherols would have been more pronounced if smaller quantities of vitamin A had been employed, as Hickman et al. ('44) have stated that the A–E synergism is more active at lower doses of vitamin A.

These experiments confirm the previous reports of Moore ('40) and Bacharach ('40) that tocopherols increase the storage of vitamin A in the liver of the rat when treated for long periods; however, we have been unable to demonstrate any effect of tocopherols when the rats are treated with vitamin A for only 3 days.

SUMMARY

1. Growth studies indicated that when vitamin A was fed at a daily level of 2.04 units to rats receiving a vitamin A–E free diet, the addition of tocopherols caused a further increase in weight.

2. Variable growth responses to comparable supplements of vitamin A were obtained when different types of vegetable oil were included in the U.S.P. vitamin A free diet. When the U.S.P. diet containing either olive or cottonseed oil was used, daily feeding of 0.3 mg of tocopherols increased the weight gain promoted by a vitamin A supplement but the addition of tocopherols to a corn oil diet had no effect on the growth of rats receiving vitamin A.

3. The supplementary effect of tocopherol administration on growth was apparent when vitamin A was fed at low levels but this effect gradually disappeared with an increasing intake of vitamin A.

4. Tocopherols exerted a supplementary effect on vitamin A utilization whether the vitamin A and tocopherols were given

together or on separate days. A water soluble preparation of tocopherols administered by injection was also shown to increase the growth response to vitamin A.

5. The storage of vitamin A in the liver of the rat was increased when tocopherols were given with vitamin A for an extended period.

LITERATURE CITED

BACHARACH, A. L. 1940 The effect of ingested vitamin E (tocopherol) on vitamin A storage in the liver of the albino rat. Quart. J. of Pharm. Pharmacol., *13:* 138.

DATTATREYA RAO, S. 1945 The influence of vitamin E on the utilization of carotene from oils. Nature, *156:* 449.

DAVIES, A. W., AND T. MOORE 1941 Interaction of vitamin A and E. Nature, *147:* 794.

GRIDGEMAN, N. T. 1944 The Estimation of Vitamin A. Lever Brothers and Unilever Ltd., Port Sunlight, Cheshire.

GUGGENHEIM, K. 1944 The biological value of carotene from various sources and the effect of vitamin E on the utilization of carotene and of vitamin A. Biochem. J., *38:* 260.

HARRIS, P. L., M. W. KALEY AND K. C. D. HICKMAN 1944 Covitamin studies. II. The sparing action of natural tocopherol concentrates on carotene. J. Biol. Chem., *152:* 313.

HICKMAN, K. C. D., P. L. HARRIS AND M. R. WOODSIDE 1942 Interrelationships of vitamins A and E. Nature, *150:* 91.

HICKMAN, K. C. D., M. W. KALEY AND P. L. HARRIS 1944 Covitamin studies. I. The sparing action of natural tocopherol concentrates on vitamin A. J. Biol. Chem., *152:* 303.

JONES, J. H., AND C. FOSTER 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, *24:* 245.

KEMMERER, A. R., G. S. FRAPS AND JEANNE DE MOTTIER 1947 Effect of xanthophylls, chlorophylls, sulfasuxidine and α-tocopherol on the utilization of carotene by rats. Arch. Biochem., *12:* 135.

LEMLEY, J. M., R. A. BROWN, O. D. BIRD AND A. D. EMMETT 1947 Absorption and storage of vitamin A in the liver of the rat. J. Nutrition, *33:* 53.

MOORE, T. 1940 The effect of vitamin E deficiency on vitamin A reserves of the rat. Biochem. J., *34:* 1321.

POPPER, H., AND B. W. VOLK 1944 Absorption of vitamin A in the rat. Arch. Path., *38:* 71.

QUACKENBUSH, F. W., R. P. COX AND H. STEENBOCK 1942 Tocopherol and the stability of carotene. J. Biol. Chem., *145:* 169.

SANDERS, R., A. BEATY AND M. DODD 1944 Effect of vitamin E on vitamin A utilization. Abstracts, Cleveland Meeting, Am. Chem. Soc., April, 1944.

SHERMAN, W. C. 1941 Activity of alpha tocopherol in preventing antagonism between linoleic and linolenic esters and carotene. Proc. Soc. Exp. Biol. Med., *47:* 199.

METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

XIII. THE UTILIZATION OF NIACIN DURING LACTATION [1]

MARGARET CORYELL, CHARLOTTE RODERUCK, MARY ELLEN HARRIS, SOL MILLER, MARJORIE M. RUTLEDGE, HAROLD H. WILLIAMS [2] AND ICIE G. MACY

Research Laboratory, Children's Fund of Michigan, Detroit

(Received for publication March 27, 1947)

Following the discovery by Elvehjem, Madden, Strong and Woolley ('38) that niacin could cure canine blacktongue, the counterpart of human pellagra, this vitamin has been used extensively to treat pellagra and associated deficiency states in human beings. Since niacinamide is a component of coenzymes I and II, it has been generally assumed that its metabolism is closely associated with tissue respiratory mechanisms involving these pyridine nucleotides.

Hamilton and Hogan ('44) found that niacin and choline were necessary for successful lactation in the hamster but little has been done to test any possibility of a relationship between niacin metabolism and lactation. In efforts to expand available knowledge of the composition of human milk and the metabolism of women during the reproductive cycle, we have determined the amounts of niacin in the 24-hour intakes of food, secretions of milk and excretions of urine by healthy mothers who were successfully nursing their infants.

[1] The investigation represented in part by this paper was partially supported by a grant from The Nutrition Foundation, Inc., and was made possible by the cooperation of J. P. Pratt, Chief of the Department of Obstetrics; B. M. Hamil, Department of Pediatrics; Elizabeth Moran, Director of Nurses, and Annie Lou Wertz, dietitian, all of the Henry Ford Hospital, Detroit.

[2] Now professor, Department of Biochemistry, Cornell University, Ithaca, N. Y.

PROCEDURE

Niacin intake, secretion in milk and excretion in urine were studied with 7 healthy nursing mothers during the first 10 days postpartum and with 9 women during periods of 5 consecutive days 2 to 10 months postpartum. Earlier papers have presented the organization of the study (Macy, Williams, Pratt and Hamil, '45) the method of manual expression used in collecting the complete 24-hour secretions of milk (Davies, '45), the diets and preparations of the food and milk (Kaucher, Moyer, Richards, Williams, Wertz and Macy, '45), urine (Roderuck, Williams and Macy, '46), and the concentration of niacin in immature and mature human milk (Coryell, Harris, Miller, Williams and Macy, '45).

METHODS

The preparation of the milk samples for assay and the microbiological procedure employing *Lactobacillus arabinosus* have been described (Coryell et al., '45).

In each 5-day period of study all subjects received diets comparable in food distribution but adjusted in level so that the appetites of the women were satisfied. Aliquots of each serving of every food eaten during a 5-day period were collected, combined, ground and thoroughly mixed. For the analyses for niacin, pantothenic acid and biotin,[3] a 400-gm quantity of the food composite was homogenized in a Waring Blendor, transferred quantitatively to a 500-ml volumetric flask and diluted to volume. Duplicate aliquots (5 ml) were pipetted into 250 ml Erlenmeyer flasks, 50 ml of 2 *N* sulfuric acid added to each flask and the mixtures autoclaved for 3 hours at 15 pounds pressure. After cooling, the contents were brought to pH 5.0 with sodium hydroxide, diluted to 500 ml with distilled water, and filtered through Whatman no. 42 paper. The dilution of the filtrate was made 1:200 or 1:250 before assay. Quantities of 1, 2, 3, 4 and 5 ml of the diluted filtrates, in duplicate, were placed in 10 assay tubes

[3] Papers presenting the results for biotin and pantothenic acid are being prepared.

and standard amounts of basal medium added. After autoclaving the tubes were inoculated, incubated, and the growths measured turbidimetrically and titrimetrically as described for milk.

Control experiments with the food composites showed that hydrolysis with hydrochloric or sulfuric acids of 1 *N* or more gave maximum niacin values, that is, values equivalent to those obtained with alkali hydrolysis. Krehl and Strong ('44) and Krehl, Elvehjem and Strong ('44) have shown the presence of a naturally occurring precursor of niacin in certain biological materials, notably cereals, which is apparently utilized by the animal organism but not by *L. arabinosus.* However, the question of its complete utilization by the animal organism is controversial. This precursor, presumably an alkyl ester of niacin, is labile to alkali or strong acids but not appreciably to weak acids (0.1 *N*). With the food composites analyzed, autoclaving with 0.1 *N* hydrochloric acid for 30 minutes gave values which averaged 9% lower than those obtained after autoclaving with 1 *N* or stronger acids for the same time. Since the use of 2 *N* sulfuric acid for 3 hours was found to be optimum for the release of bound biotin[4] and gave values for niacin identical with those obtained using 1 *N* acid for 30 minutes, the hydrolysis with stronger acid was used in the determination of both vitamins. Sodium hydroxide was employed to neutralize the sulfuric acid, since the values were found not to differ from those obtained using barium hydroxide.

Aliquots of the 24-hour collections of urine were filtered and the pH adjusted to 6.5 to 7.0 then diluted to suitable concentrations, usually 1:20, and assayed by the microbiological procedure described for milk. The values obtained by this method represent niacin, niacinamide, and any nicotinuric acid present. As with the milk, no attempt was made to differentiate between these 3 metabolites in the urine.

[4] See footnote 3, p. 220.

Knowledge concerning the fluorescent nicotinic acid metabolite in the urine, noted in 1940 by Najjar and Wood and in 1943 designated as F_2, was limited until Huff and Perlzweig ('43a) isolated N^1-methylnicotinamide chloride [5] from urine and identified it as "F_2" or its immediate precursor. Semiquantitative values for N^1-methylnicotinamide in urine were obtained for 14 5-day periods by a method similar to that of Huff and Perlzweig ('43b), in conjunction with thiamine determinations which have been published (Roderuck, Williams and Macy, '45, '46). The blank employed was the sulfite blank of Mason and Williams ('42). The eluate was treated with alkaline ferricyanide according to the thiochrome procedure. According to Najjar and Ketron ('44) the sulfite destroys some "F_2" and the ferricyanide converts "F_2" (a pyridine derivative) into pyridone, which possesses only 21% of the original fluorescence. Control experiments showed the destruction of N^1-methylnicotinamide by the sulfite to be rather variable, averaging 23%. The decrease in fluorescence as a result of ferricyanide treatment was uniform, averaging 76%, whether standard solutions of N^1-methylnicotinamide or urine samples previously treated with sulfite were employed. If an average of 23% of the N^1-methylnicotinamide in the urine was destroyed by the sulfite treatment and of the remaining 77% only 24% of the resulting fluorescence remained after ferricyanide treatment, the values obtained for N^1-methylnicotinamide in the urine, using the sulfite, are 19% lower than with those which would be obtained using the more commonly employed water blank. Since this work was completed a more reliable method of analysis has been published by Huff and Perlzweig ('47).

RESULTS AND DISCUSSION

The volumes of milk and urine collected each 24 hours during the first 10 days postpartum and their niacin contents are given in table 1. Values for N^1-methylnicotinamide in

[5] The N^1-methylnicotinamide chloride against which the quinine sulfate solutions were standardized was provided by Dr. W. A. Perlzweig, Duke University Medical School, Durham, N. C.

TABLE 1

Niacin excretion in urine and secretion in milk during first 10 days postpartum.[1]

INTERVAL POSTPARTUM	VOLUMES		NIACIN		N¹-METHYL-NICOTINAMIDE CHLORIDE
	Milk	Urine	Milk	Urine	
days	*ml*	*ml*	*mg*	*mg*	*mg*
	Subject: V.L.				
1	30	1757	0.04	1.23	1.4
2	56	2686	0.04	1.26	..
3	353	2759	0.22	1.33	1.6
4	794	1823	0.41	1.48	1.2
5	844	1847	0.50	1.41	1.8
6	955	2710	0.64	1.21	2.6
7	1047	2841	0.83	1.45	1.8
8	1098	2066	1.24	1.15	4.1
9	1118	2151	1.53	1.64	2.8
10	1200	1669	2.06	1.47	1.6
	Subject: V.S.				
1	6	1869		0.97	1.7
2	92	1874	0.07	0.88	1.1
3	420	2887	0.24	0.86	3.7
4	600	2063	0.38	0.83	1.9
5	697	2065	0.51	0.92	4.5
6	756	1502	0.77	0.67	3.1
7	818	2524	1.25	0.92	2.0
8	837	2237	1.82	0.73	2.1
9	932	1984	2.38	0.78	2.7
10	924	2287	2.78	0.68	1.7
	Subject: L.F.				
1	71	2590	0.05	1.32	
2	200	1275	0.12	0.94	
3	733	2420	0.66	1.31	
4	1122	2075	0.85	1.12	
5	1441	1674	1.21	1.12	
6	1501	1718	1.56	1.23	
7	1596	1340	2.42	1.18	
8	1638	1810	3.44	1.25	
9	1676	1374	4.47	0.44	
10	1872	2220	5.69	1.31	
	Subject: V.G.				
1	82	2067	0.07	0.69	
2	177	1689	0.11	0.72	
3	849	1775	0.49	0.78	
4	1413	1323	0.82	0.66	
5	1471	1303	0.81	0.83	
6	1782	1148	1.07	0.71	
7	1630	1230	1.28	0.71	
8	1895	1237	2.02	0.68	
9	1828	1104	2.56	0.61	
10	1770	1075	2.79	0.63	

INTERVAL POST-PARTUM	VOLUMES		NIACIN	
	Milk	Urine	Milk	Urine
days	*ml*	*ml*	*mg*	*mg*
	Subject: V.K.			
1	9	3086	0.01	0.74
2	90	3651	0.06	0.79
3	484	2596	0.30	0.65
4	547	2427	0.48	0.70
5	560	2331	0.65	0.67
6	663	2249	0.98	0.70
7	781	3380	1.34	0.90
8	775	3840	1.80	0.84
9	794	3115	2.22	0.78
10	797	3328	2.31	0.83
	Subject: J.M.			
1	35	2182	0.03	0.87
2	385	1386	0.19	0.89
3	870	1519	0.52	0.85
4	1011	1201	0.74	0.76
5	1121	1283	0.99	0.87
6	1125	1172	1.33	0.73
7	1287	1161	1.90	0.97
8	1136	1520	2.14	0.96
9	1258	....	2.64	...
10	1336	1553	3.31	0.95
	Subject: C.O.			
1	16	1864	0.01	0.60
2	100	939	0.09	1.01
3	335	1511	0.21	0.99
4	595	....	0.41	...
5	725	....	0.70	...
6	821	....	0.84	...
7	798	1470	1.05	0.84
8	950	1127	1.77	0.90
9	931	1191	1.92	0.97
10	660	....	1.65	...
	Subject: M.B.			
9	1189	1035	4.28	0.59
	Subject: E.L.			
8	688	677	...	0.76
	Subject: M.S.			
7	880	1355	1.20	0.58
	Subject: G.S.			
7	1017	1981	1.04	0.84
	Subject: F.W.			
7	953	1032	1.30	0.81

[1] The first day postpartum was variable to the extent of differences in the times at which the women delivered. For subjects delivered after 12 M. the first day began the following morning. The first day for C.O. was 22 hours; for L.F. 15 hours. V.G. was delivered 8 hours before the beginning of the first day postpartum; V.K. 17 hours; V.L. 5 hours; J.M. 16 hours; and V.S. 7 hours.

the urine of 2 women during the puerperium also are given in table 1. For each 5-day period of study table 2 presents the average daily energy intake, milk and urine volumes, and the amounts of niacin in food intake, in the milk and in the urine. The amounts of N^1-methylnicotinamide determined in urine during certain periods are also included in table 2. For intake of niacin both the values obtained by hydrolysis with weak acid, and those for niacin plus its precursor, obtained by hydrolysis with strong acid, are given in the table.

The food eaten by the mothers during the first 10 days postpartum provided averages for the 5-day periods of 13 to 22 mg of niacin and its precursor per day. The rapid increases in the amounts of niacin secreted daily in milk portray both the increases in concentration (Coryell, Harris, Miller, Williams and Macy, '45) and volume during the puerperium. Niacin in the urine varied from day to day for the individual women but each subject seemed to have a characteristic level of excretion. During the puerperium neither secretion in milk nor excretion in urine show relationship with intake; nor do the daily amounts of niacin in urine show a relationship to the volumes of urine or milk, or the quantity of the vitamin in the milk.

For all of the women studied, during the puerperium and while they were secreting mature milk, the average daily intakes of niacin and its precursor during the 5-day periods ranged from 13.0 to 23.4 mg as determined for the food eaten, and the energy intakes ranged from 2256 to 3559 cal. per day, thus the niacin intakes per 1000 cal. ranged from 5.0 to 7.8 mg. The values for niacin in milk (table 2) show a general relationship to milk volume rather than to intake and illustrate the wide range of normal variation in the composition of human milk from different mothers and from the same mother at different times. Greater amounts of niacin were excreted in the urine during the 2 5-day periods immediately postpartum than during periods later in lactation.

The percentage of the average daily niacin intakes secreted in milk ranged from 1 to 3 for the first 5 days postpartum,

TABLE 2

Average daily niacin intakes, excretion in urine and secretion in milk during 31 5-day study periods.

	INTERVAL POST-PARTUM	ENERGY INTAKE	VOLUMES		NIACIN						N¹-METHYL-NICOTINAMIDE CHLORIDE
					Intake		Milk		Urine		Urine
			Milk	Urine	Total [1]	Free					
	days	*cal.*	*ml*	*ml*	*mg*	*mg*	*mg*	*% intake*	*mg*	*% intake*	*mg*
M.B.	72–76	2547	718	691	14.8	13.4	1.34	9	0.35	2	
L.F.	1–5	2850	713	2007	18.5	16.5	0.58	3	1.16	6	
	6–10	2898	1657	1692	16.1	14.1	3.52	22	1.08	7	
V.G.	1–5	2475	798	1631	13.6	12.4	0.46	3	0.74	5	
	6–10	2445	1781	1159	13.2	11.8	1.95	15	0.67	5	
	78–82	....	848	1281	23.4	20.9	2.02	9	0.69	3	
	161–165	2873	901	993	19.4	17.1	1.83	9	0.50	2	0.9
	239–243	2925	681	810	17.0	15.7	1.16	7	0.48	3	4.4
	302–306 [2]	2827	394	1080	18.7	16.9	0.64	3	0.51	3	4.6
V.K.	1–5	2403	338	2818	13.0	11.7	0.30	2	0.71	5	
	6–10	2381	762	3182	15.0	13.3	1.73	12	0.81	5	
	95–99	2822	647	2804	14.6	13.6	1.30	9	0.52	4	0.8
	144–148 [2]	2806	325	1447	14.7	13.6	0.56	4	0.48	3	2.4
V.L.	1–5	3167	415	2174	18.8	16.6	0.24	1	1.34	7	1.5
	6–10	3365	1084	2287	22.6	20.8	1.26	6	1.38	6	2.6
	68–72	3275	789	2849	21.6	19.0	1.77	8	1.08	5	4.0
	152–156	3253	680	1756	21.2	18.6	1.17	6	0.53	2	4.0
J.M.	1–5	3269	684	1514	16.4	15.4	0.49	3	0.85	5	
	6–10	3412	1228	1352	18.8	18.3	2.26	12	0.90	5	
	75–79	3225	708	2265	20.2	19.4	1.57	8	0.67	3	4.7
	173–179 [2]	3559	268	2003	20.5	19.2	0.52	2	0.68	3	5.5
C.O.	1–5	2256	354	1438	13.4	12.6	0.28	2	0.87	6	
	6–10	2452	832	1263	13.6	13.8	1.44	10	0.90	7	
B.S.	85–89	2762	1020	1347	19.1	17.6	1.71	9	0.56	3	
	204–208	2825	913	1356	18.9	16.6	2.00	10	0.63	3	
	259–263	2918	676	1219	20.6	17.8	1.40	7	0.70	3	
M.S.	58–62	2735	947	939	18.4	16.7	1.31	7	0.48	3	
G.S.	80–84	2653	899	1078	20.8	19.5	1.65	8	0.62	3	
V.S.	1–5	3278	363	2152	18.0	16.2	0.24	1	0.89	5	2.6
	6–10	3474	853	2107	20.1	18.6	1.80	9	0.76	4	2.3
	70–74 [2]	3350	304	1852	20.1	16.8	0.86	4	0.63	3	8.8

[1] Total indicates free + precursor. Free niacin was obtained with 0.1 *N* HCl.

[2] Lactation terminating.

with 5 to 7% of the intake being excreted in urine. For the next 5 days niacin in the milk ranged from 6 to 22% of the intake but the average daily excretions in urine were comparable to those of the preceding period. During the periods of mature milk production averages of only 2 to 10% of the intakes were secreted in milk, while only 2 to 5% was found in the urine. The data for healthy women receiving good diets do not indicate a relationship between intake and secretion in milk or excretion in urine, between secretion in milk and excretion in urine, or between urine volume and niacin content. That the data represent the physiologic performance of average healthy women whose nutritional status was good before and after delivery is emphasized by the values for V.S. who throughout pregnancy and lactation, except for the intervals of study, was ingesting daily, in addition to a good diet, 6250 I.U. of vitamin A, 3.6 mg of thiamine, 2.0 mg of riboflavin, 1000 I.U. of vitamin D, 10 mg of niacin and 0.5 mg of pantothenic acid.

The low values for niacin in the urine are in agreement with the findings of most investigators that "niacin" is quite low and uniform and has little relationship with intake, at least within a rather wide range of the latter. The urine "niacin" values include niacinamide and any nicotinuric acid (a conjugate of niacin with glycine) that may be present, since *L. arabinosus* can utilize all 3 forms. Based on chemical differentiation, the presence of nicotinuric acid in human urine under normal conditions has been controversial.

Recently Johnson ('45) developed a microbiological procedure using *Leuconostoc mesenteroides* in conjunction with *L. arabinosus* to differentiate between niacin, its amide, and the glycine conjugate. Using this method he was unable to find any nicotinuric acid in the urine of normal subjects, either while they were ingesting average diets or following supplementation with 50 mg of niacinamide per day for 5 days (Johnson, Hamilton and Mitchell, '45). With daily diets estimated to contain an average of 21.1 mg of niacin, he found an average daily urinary output of 1.15 mg of niacin

and its amide. This value is comparable to the 1.1 to 1.5 mg obtained by Sarett, Huff and Perlzweig ('42) using a chemical procedure.

The values determined for N^1-methylnicotinamide in the urine of the nursing mothers, averaged 3.5 mg per day. The niacin equivalent, 2.5 mg, calculated with the factor 0.713, represented 13% of the intake. The totals of the niacin in the milk and urine, and the niacin equivalents from N^1-methylnicotinamide in urine accounted for approximately 23% of the intake, on the average. If the N^1-methylnicotinamide values are increased by 19% for the residual N^1-methylnicotinamide in the sulfite blank (see procedure) the average value would be increased to 4.3 mg, increasing the average percentage of intake niacin accounted for from 23 to 26. The 74% of the food niacin unaccounted for would strongly indicate either the complete breakdown of a large fraction of the exogenous niacin by the body or the presence in urine of end-products of niacin metabolism which have not been recognized. This explanation has been suggested by Perlzweig and Huff ('45).

Values in the literature for average N^1-methylnicotinamide elimination in the urine of normal adults eating average diets vary, in niacin equivalents, from 2.3 mg (Mickelson and Erickson, '45) to 18.7 mg (Johnson, Hamilton and Mitchell, '45). The lack of agreement between different reports may be attributable in part to the methods of analysis. Nevertheless, one of the most consistent findings is the rather wide variation between individuals and between different days with the same individual.

Numerous factors besides niacin and nicotinamide intake undoubtedly affect the excretion of this metabolite. The availability of methyl groups in the body and the condition of the liver have been suggested (Perlzweig, Bernheim and Bernheim, '43; Najjar, Hall and Deal, '45), as well as body activity (Ellinger and Coulson, '44). The upper value of the range for average daily urinary N^1-methylnicotinamide was that of subject V.S. at 70 to 74 days postpartum. The 10 mg

of niacin included in the vitamin supplements taken by V.S. each day except during the study periods was equal to 55% of the average amount in the food eaten during the period of investigation. The values for her excretion of N^1-methylnicotinamide, as the chloride, were 16.65, 7.68, 5.11, 5.48 and 8.86, mg, respectively, for the 5 days. The relatively large excretion on the first day may have resulted from the supplement prior to initiation of the study. However, the values for niacin in the milk and in the urine on the first day were either equal to or less than the corresponding values for the following 4 days.

The possibility of synthesis by intestinal flora as a factor in the variable results cannot be disregarded. Ellinger and Benesch ('45) have shown a marked decrease in the excretion of nicotinamide methochloride (N^1-methylnicotinamide) by the subjects after ingestion of the "sterilising" drugs, sulfaguanidine or succinyl sulfathiazole. However, ingestion by infants of 1 gm of *l*-tryptophane and by adults of 5 gm of *dl*-tryptophane has been found to produce a "prompt and marked increase in the urinary excretion of nicotinic acid derivatives, chiefly in the methylated form" (Perlzweig, Rosen, Levitas and Robinson, '47). A comprehensive study of young women by Oldham, Davis and Roberts ('46) of the intakes and excretions of certain of the B vitamins reported values for fecal niacin ranging from 0.9 to 1.7 mg per day.

The data emphasize that greater understanding of the dietary requirements and metabolism of pregnant and lactating women is contingent upon obtaining much clearer and more complete knowledge of the physiology of milk production and secretion. As the values from our investigations are brought together, impressive evidence of the unique physiology of lactation accumulates. Many factors are interrelated in producing the over-all body changes consequent to lactation and other reports containing data for the same group of healthy nursing mothers have demonstrated that for vitamin C (Munks, Kaucher, Moyer, Harris and Macy, '47), thiamine (Roderuck, Williams and Macy, '46), ribo-

flavin (Roderuck, Coryell, Williams and Macy, '46), and vitamin A (Lesher, Brody, Williams and Macy, '47), body performance during lactation cannot be interpreted in terms of results obtained with non-pregnant, non-lactating subjects.

SUMMARY

The niacin intake for 5-day periods and output in 24-hour collections of milk and urine were determined for normal multiparas during the first 10 days postpartum and at various intervals during mature milk production. The intakes of the women were comparable qualitatively but the quantity was determined by appetite. With an average daily intake of 16.5 mg of niacin the average secretion in milk per 24 hours increased from 0.04 mg on the first day postpartum to 2.94 mg on the tenth day. Excretion in the urine ranged from 0.92 to 0.98 mg.

The average volume of mature milk secreted per day during each of 17 5-day periods ranged from 268 to 1020 ml. The average daily niacin content of the milk ranged from 0.52 to 2.02 mg. From 0.35 to 1.08 mg of niacin were excreted in urine. During 10 5-day periods the average daily urinary excretion of N^1-methylnicotinamide ranged from 0.8 to 8.8 mg averaging 4.0 mg. Of the daily niacin intakes during mature milk production, averages of 7 and 3%, respectively, appeared in the milk and urine as niacin.

LITERATURE CITED

Coryell, M. N., M. E. Harris, S. Miller, H. H. Williams and I. G. Macy 1945 Human milk studies. XXII. Nicotinic acid, pantothenic acid and biotin contents of colostrum and mature human milk. Am. J. Dis. Children, *70:* 150.

Davies, V. 1945 Human milk studies. XXI. A simple technic for the manual expression of mothers' milk. Am. J. Dis. Children, *70:* 148.

Ellinger, P., and R. Benesch 1945 Biosynthesis of "nicotinamide" in the human gut. Lancet, *1:* 432.

Ellinger, P., and R. A. Coulson 1944 The urinary elimination of nicotinamide methochloride by man. Biochem. J., *38:* 265.

Elvehjem, C. A., R. J. Madden, F. M. Strong and D. W. Woolley 1938 The isolation and identification of the anti-black tongue factor. J. Biol. Chem., *123:* 137.

HAMILTON, J. W., AND A. G. HOGAN 1944 Nutritional requirements of the Syrian hamster. J. Nutrition, *27:* 213.

HUFF, J. W., AND W. A. PERLZWEIG 1943a N^1-methylnicotinamide, a metabolite of nicotinic acid in the urine. J. Biol. Chem., *150:* 395.

———— 1943b A rapid method of estimating N^1-methylnicotinamide in urine. J. Biol. Chem., *150:* 483.

———— 1947 The fluorescent condensation product of N^1-methylnicotinamide and acetone. II. A sensitive method for the determination of N^1-methylnicotinamide in urine. J. Biol. Chem., *167:* 157.

JOHNSON, B. C. 1945 The microbiological determination of nicotinic acid, nicotinamide, and nicotinuric acid. J. Biol. Chem., *159:* 227.

JOHNSON, B. C., T. S. HAMILTON AND H. H. MITCHELL 1945 The excretion of nicotinic acid, nicotinamide, nicotinuric acid, and N^1-methylnicotinamide, by normal individuals. J. Biol. Chem., *159:* 231.

KAUCHER, M., E. Z. MOYER, A. J. RICHARDS, H. H. WILLIAMS, A. L. WERTZ AND I. G. MACY 1945 Human milk studies. XX. The diet of lactating women and the collection and preparation of food and human milk for analysis. Am. J. Dis. Children, *70:* 142.

KREHL, W. A., AND F. M. STRONG 1944 Studies on the distribution, properties, and isolation of a naturally occurring precursor of nicotinic acid. J. Biol. Chem., *156:* 1.

KREHL, W. A., C. A. ELVEHJEM AND F. M. STRONG 1944 The biological activity of a precursor of nicotinic acid in cereal products. J. Biol. Chem., *156:* 13.

LESHER, M., J. K. BRODY, H. H. WILLIAMS AND I. G. MACY 1947 Metabolism of women during the reproductive cycle. X. The utilization of vitamin A during lactation. J. Am. Diet. Asso., *23:* 211.

MACY, I. G., H. H. WILLIAMS, J. P. PRATT AND B. M. HAMIL 1945 Human milk studies. XIX. Implications of breast feeding and their investigation. Am. J. Dis. Children, *70:* 135.

MASON, H. L., AND R. D. WILLIAMS 1942 Determination of thiamine in urine by the thiochrome method; estimation of the blank. J. Biol. Chem., *146:* 589.

MICKELSON, O., AND L. L. ERICKSON 1945 The F_1 excretion of normal men on different levels of niacin intake. Proc. Soc. Exp. Biol. Med., *58:* 33.

MUNKS, B., M. KAUCHER, E. Z. MOYER, M. E. HARRIS AND I. G. MACY 1947 Metabolism of women during the reproductive cycle. XI. Vitamin C in diets, breast milk, blood and urine of nursing mothers. J. Nutrition, *33:* 60.

NAJJAR, V. A., AND K. C. KETRON 1944 An improved thiochrome method for the determination of thiamin in urine. J. Biol. Chem., *152:* 579.

NAJJAR, V. A., AND R. W. WOOD 1940 Presence of a hitherto unrecognized nicotinic acid derivative in human urine. Proc. Soc. Exp. Biol. Med., *44:* 386.

NAJJAR, V. A., R. S. HALL AND C. C. DEAL 1945 Methylation of nicotinamide; simple and practical test for liver function (preliminary note). Bull. Johns Hopkins Hosp., *76:* 83.

OLDHAM, H. G., M. V. DAVIS AND L. J. ROBERTS 1946 Thiamine excretions and blood levels of young women on diets containing varying levels of the B vitamins, with some observations on niacin and pantothenic acid. J. Nutrition, *32:* 163.

PERLZWEIG, W. A., M. L. C. BERNHEIM AND F. BERNHEIM 1943 The methylation of nicotinamide by rat liver in vitro. J. Biol. Chem., *150:* 401.

PERLZWEIG, W. A., AND J. W. HUFF 1945 The fate of N^1-methylnicotinamide in man. J. Biol. Chem., *161:* 417.

PERLZWEIG, W. A., F. ROSEN, N. LEVITAS AND J. ROBINSON 1947 The excretion of nicotinic acid derivatives after ingestion of trypophane by man. J. Biol. Chem., *167*. 511.

RODERUCK, C., M. N. CORYELL, H. H. WILLIAMS AND I. G. MACY 1946 Metabolism of women during the reproductive cycle. IX. The utilization of riboflavin during lactation. J. Nutrition, *32:* 267.

RODERUCK, C. E., H. H. WILLIAMS AND I. G. MACY 1945 Human milk studies. XXIII. Free and total thiamine contents of colostrum and mature human milk. Am. J. Dis. Children, *70:* 162.

———— 1946 Metabolism of women during the reproductive cycle. VIII. The utilization of thiamine during lactation. J. Nutrition, *32:* 249.

SARETT, H. P., J. W. HUFF AND W. A. PERLZWEIG 1942 Studies in nicotinic acid metabolism. I. The fate of nicotinic acid in man. J. Nutrition, *23:* 23.

A CHICK GROWTH FACTOR IN COW MANURE

V. RELATION TO QUANTITY AND QUALITY OF SOYBEAN OIL MEAL IN THE DIET

MAX RUBIN[1] AND H. R. BIRD

Bureau of Animal Industry, U. S. Department of Agriculture, Agricultural Research Center, Beltsville, Maryland

(Received for publication February 27, 1947)

It has been reported (Rubin and Bird, '46a, b) that an all-plant protein diet containing 35% of commercially heated soybean oil meal produced suboptimal growth of chickens unless supplemented with fish meal, cow manure or extracts of cow manure.

This diet was adequate in meeting the requirements for the essential amino acids according to Almquist's data ('46). It was shown to be complete in all of the chemically characterized vitamins by Whitson et al. ('45) and in the unidentified factors (Rubin and Bird, '46a), except for the growth factor which was obtained as a concentrate from extracts of cow manure (Rubin and Bird, '46b).

Whitson et al. ('46a) described the detrimental effect of high levels of soybean oil meal in the diet of hens on the hatchability of their eggs. The diets, although unusually high in protein, apparently were not toxic to the hens nor did they adversely affect egg production. It appeared that for each increment of soybean oil meal in the breeder diet, an additional increment of a substance which interfered with embryonic development was deposited in the egg. Ham and Sandstedt ('44) showed that there is a growth inhibitor in raw soybean oil meal presumably identical with the trypsin inhibitor described by them and crystallized by Kunitz ('46).

[1] Resigned.

They also indicated that the growth inhibitor is heat labile. Therefore, it would probably be destroyed in commercially heated soybean oil meal. Furthermore, the inhibitor was shown to be a protein and hence its absorption through the gut, per se, appears very unlikely. Therefore, the low hatchability reported by Whitson et al. ('46a) is probably due to some other cause than this heat labile inhibitor of trypsin and of growth.

Bird and Mattingly ('45) reported that they obtained optimum growth with soybean and corn diets which were supplemented with 0.2% methionine. Evans and McGinnis ('46) obtained improved chick growth with soybean diets supplemented with methionine. Bird et al. ('47) showed that the addition of crystalline methionine to the 35% soybean oil meal diet produced as good growth as did the growth factor of cow manure provided this growth factor had been present in the diet of the dams. Methionine was less effective when fed to the progeny of hens whose diet was deficient in the growth factor. This partial effectiveness of methionine is difficult to explain since the basal diet is supposed to contain adequate quantities of the essential amino acids. Some interrelation of the growth factor and methionine is indicated. The growth factor might conceivably facilitate the liberation of methionine from soybean protein in the digestive tract, it might function in the metabolism of methionine after absorption, or it might have some entirely different metabolic function which could be partially performed by methionine.

The literature on the value of cystine and methionine as supplements to raw and heated soybean oil meal has been reviewed by Barnes and Maack ('43). Evans and McGinnis ('46) recently reported on the availability of the sulfur amino acids of raw and autoclaved soybean oil meal alone and when supplemented with methionine. Since the sulfur-containing amino acids improve the nutritional value of either raw or heated soybean oil meal, it was of interest to determine what effect the growth factor of cow manure would have on the

nutritional value of all-plant-protein diets in which raw soybean oil meal was the major source of protein.

The objects of the experiments described in this paper were first, to determine whether the inhibition of embryonic development by high levels of commercially heated soybean oil meal would be paralleled by inhibition of the growth of chicks fed an all-plant-protein diet containing high levels of such soybean oil meal, and second, to determine the effect of the growth factor of cow manure or methionine on the growth of chicks fed high levels of heated or ordinary levels of raw soybean oil meal.

METHODS AND RESULTS

The chicks in all of the experiments except 6 and 7 were the progeny of crossbred dams (Rhole Island Red X Barred Plymouth Rock) and New Hampshire cocks. The chicks in experiments 6 and 7 were the progeny of Rhode Island Red dams and Barred Plymouth Rock cocks. All chicks were fed the basal diet during the first 2 weeks after hatching. At 2 weeks of age they were divided into experimental groups. The distribution into groups was made according to weight; the middle weight group was used and the light and heavy chicks were discarded. All experiments were terminated when the chickens were 6 weeks old.

The percentage composition of the basal diet was as follows: yellow corn 38.0, barley 20.0, alfalfa leaf meal 3.0, soybean oil meal 35.0, butyl fermentation solubles (containing 250 μg of riboflavin per gm) 0.6, steamed bone meal 1.5, lime stone 1.0, salt (96% NaCl, 4% $MnSO_4 \cdot 4\ H_2O$) 0.7, and vitamins A and D feeding oil (400 A.O.A.C. units of vitamin D, 2000 U.S.P. units of vitamin A per gm) 0.2. One mg of nicotinic acid was added to each 100 gm of this diet.

When this diet was modified to contain 25% of soybean oil meal, corn replaced 10% of the soybean oil meal of the basal diet. The diets containing 50% soybean oil meal were formulated by increasing the level of this material and decreasing the level of barley. In the 70% soybean oil meal diet,

this material replaced all of the barley and a portion of the corn. In the diets containing raw soybean oil meal or soybean flour, this material replaced the heated soybean oil meal of the basal diet. When other supplements were used, these replaced the corn of the basal diet. A sample of raw soybean oil meal was obtained from each of 2 processors, and from a third processor samples of raw and hydrolysed soybean flour were obtained.

Except in experiment 6, all the chicks were the progeny of hens which had been fed an all-plant-protein diet for several months. Therefore, they were well depleted of the growth factor of cow manure.

Experiments 1 to 5, the results of which are summarized in table 1, were undertaken to determine how well chicks would grow when fed high levels of soybean oil meal with or without supplements of the growth factor or of methionine. Experiments 1, 2 and 3, were conducted in batteries and 4 and 5 in a brooder house, the birds being brooded on the floor under electric hovers.

The results of experiment 1 show that the best growth was obtained with the 35% soybean oil meal diet. The chickens fed the 25% soybean oil meal diet did not grow quite so well. A marked inhibition of growth occurred in the group fed the 70% soybean oil meal diet in spite of the fact that the diet was supposedly complete and had an excess of essential amino acids.

Experiment 2 was designed to study the effect of the growth factor of cow manure when added to diets containing large quantities of soybean oil meal. In experiment 2, the usual stimulation in growth occurred when the basal diet containing 35% soybean oil meal was supplemented with the acid precipitate fraction of an extract of cow manure (Rubin and Bird, '46b). A depression in growth occurred when the birds were fed the diet containing 50% soybean oil meal; growth inhibition was even more marked when the diet contained 70% soybean oil meal. Remarkable results were obtained with both of these high soybean oil meal diets when they were

supplemented with the acid precipitate fraction. Growth was as good as or better than that obtained in the group fed the basal diet supplemented with the growth factor from cow manure. The results of experiment 3 confirmed the results of experiment 2.

TABLE 1

Effect of supplements on growth of chickens fed different quantities of soybean oil meal.

EXPERIMENT NO.	SOYBEAN OIL MEAL IN DIET	SUPPLEMENT TO DIET	AV. WT. OF CHICKENS AT 6 WKS.	NO. OF CHICKS STARTED	NO. OF SURVIVORS
	%		gm		
1	25	None	378	12	11
	35	None	408	12	12
	70	None	256	12	10
2	35	None	361	12	11
	35	0.075% A.P.[1]	431	12	12
	50	None	295	12	8
	50	0.075% A.P.	472	12	12
	70	None	181	12	7
	70	0.075% A.P.	430	12	11
3	35	None	340	14	14
	70	None	294	14	12
	70	0.075% A.P.	406	14	14
4	35	None	422	47	47
	70	None	365	47	34
	70	0.2% dl-methionine	304	47	37
	70	0.4% dl-methionine	350	47	39
5	70	None	295	37	29
	70	None	326	37	31
	70	5% dried cow manure	467	37	33
	70	5% dried cow manure	441	37	37
	70	0.4% dl-methionine	360	37	29
	70	0.4% dl-methionine	315	37	35

[1] Acid precipitate of water extract of dried cow manure.

In experiment 4 the effect of methionine on the 70% soybean oil meal diet was studied. In experiment 5 certain diets used in experiment 4 were repeated with the addition of 2 groups fed the 70% soybean oil meal diet and supplemented with 5% cow manure. The results of both experiments show that

methionine had little or no counter-effect on the high soybean oil meal diet. On the other hand, the groups fed the diet containing cow manure grew at an optimum rate. The chicks used in all of these experiments were from the eggs of hens whose diet had been deficient in growth factor, and it would be expected, therefore, that the chicks would have shown less response to methionine than to the growth factor even if they had been fed these supplements with 35% of soybean oil meal (Bird et al., '47). The 70% level accentuated the difference between the 2 supplements to the extent that methionine produced little or no stimulus to growth in experiments 4 and 5.

TABLE 2

Effect of maternal diet on chickens fed diets high in soybean oil meal. (Experiment 6.)

MATERNAL DIET NO.	SOYBEAN OIL MEAL IN CHICK DIET	A.P.[1] IN CHICK DIET	AV. WT. AT 6 WKS.	NO. OF SURVIVORS
	%	%	*gm*	
311	35	0	238	11
311	35	0.075	425	16
312	35	0	383	20
312	70	0	315	16

[1] Same as in table 1.

Experiment 6 was designed to determine the effect of the growth factor in the maternal diet on the growth of chicks fed the high soybean oil meal diet. There were 4 groups of 20 chicks. Two groups were composed of chicks whose dams were fed a diet (diet 311, Rubin and Bird, '46b) deficient in the growth factor; the other 2 groups were composed of chicks whose dams were fed a diet (diet 312) which contained the factor. The chicks in this experiment were reared in batteries. The data are given in table 2.

In this experiment, 70% of soybean oil meal reduced the growth rate of the progeny of hens fed a "good" diet (no. 312), but these chicks, even when fed the high level of soybean oil meal, were still able, by virtue of the diet of their dams, to grow more rapidly than those fed 35% of soybean oil meal

but subject to the disadvantage of a poor maternal diet. This is evidence that the growth factor, even when stored in the chick's body, counteracted the inhibitory effect of a high soybean diet.

In all of these experiments excessive quantities of soybean oil meal in the diet caused considerable mortality which was prevented by the addition of the growth factor of cow manure.

Experiments 7 to 11, the results of which are summarized in table 3, were undertaken to determine the effectiveness of the growth factor in overcoming the deficiency of raw soybean oil meal and to compare its effectiveness with that of methionine and with the effectiveness of hydrolysis of the soybean product. The experimental groups, each consisting of 12 chicks, were reared in battery brooders, except that in experiment 8 groups of 36 chicks were brooded under electric hovers in the brooder house.

In experiment 7, the usual difference between the basal diet containing heated soybean oil meal and the same diet supplemented with the growth factor was observed. Methionine improved this basal diet but not quite so much as did the growth factor. The expected poor growth was obtained with the basal diet containing raw soybean oil meal. The growth factor improved this diet considerably but optimum growth was not achieved. Methionine in the raw soybean oil meal diet made it equal to the heated soybean oil meal basal diet.

The results of experiment 8 were essentially the same as those of the preceding one. Raw soybean oil meal no. 1 was inferior to the heated soybean oil meal. The growth factor, supplied by the dried cow manure improved the raw soybean oil meal diet considerably but did not make it equal to the diet containing heated soybean oil meal plus cow manure.

In experiment 9 a second sample of raw soybean oil meal, obtained from another processor, was compared with the first sample. This experiment confirmed the results of the first 2 experiments. About the same growth response was

obtained with the 2 different samples of raw soybean oil meal.

The second sample of raw soybean oil meal was retested in experiment 10. The results confirmed the results of the previous experiment. The growth factor improved the nutritional value of raw soybean oil meal but not to the extent obtained with heated soybean oil meal.

TABLE 3

Effect of supplements on growth of chickens fed heated and unheated soybean products as 35% of diet.

EXPERIMENT NO.	SOYBEAN PRODUCT IN DIET	SUPPLEMENT TO DIET	AV. WT. OF CHICKENS 6 WKS.
			gm
7	Heated oil meal	None	356
	Heated oil meal	0.075% A.P.[1]	440
	Heated oil meal	0.2% dl-methionine	390
	Raw oil meal, no. 1	None	323
	Raw oil meal, no. 1	0.075% A.P.	394
	Raw oil meal, no. 1	0.150% A.P.	414
	Raw oil meal, no. 1	0.2% dl-methionine	360
8	Heated oil meal	None	361
	Heated oil meal	5% dried cow manure	498
	Raw oil meal, no. 1	None	326
	Raw oil meal, no. 1	5% dried cow manure	429
9	Heated oil meal	None	290
	Heated oil meal	0.075% A.P.	419
	Raw oil meal, no. 1	None	247
	Raw oil meal, no. 1	0.15% A.P.	337
	Raw oil meal, no. 1	0.30% A.P.	345
	Raw oil meal, no. 2	None	244
	Raw oil meal, no. 2	0.135% A.P.	349
10	Heated oil meal	None	236
	Heated oil meal	0.075% A.P.	383
	Raw oil meal, no. 2	None	199
	Raw oil meal, no. 2	0.150% A.P.	332
	Raw oil meal, no. 2	0.225% A.P.	312
11	Heated oil meal	None	347
	Heated oil meal	0.075% A.P.	407
	Raw flour	None	263
	Flour hydrolysate	None	314

[1] Acid precipitate of water extract of dried cow manure.

In experiment 11, the effect of enzymatic hydrolysis of the proteins in soybean flour was studied. The flour and the hydrolysate of the flour were commercial products. Unfortunately, only enough of the hydrolysate was available for 1 experimental group; therefore, the supplementary effect of the growth factor could not be studied. The results of this experiment showed that while enzymatic hydrolysis of raw soybean flour improved it, it was not quite so good as heated soybean oil meal which was not supplemented with the growth factor. The group fed the growth factor with heated soybean oil meal was superior to all of the other groups.

DISCUSSION

The results of experiments 1 to 6 show that there is a parallelism between the effect of increasing levels of soybean oil meal on embryonic development (hatchability), as shown by Whitson et al. ('46a), and the effect on chick growth. Just as increasing quantities of soybean oil meal in the hen's diet caused a reduction in hatchability, increasing quantities of soybean oil meal in the chick's diet caused a reduction in viability and growth rate. The parallelism was also evident in the effect of cow manure. The addition of this material or concentrates prepared from it to diets containing high levels of soybean oil meal prevented the inhibition of growth and mortality which usually occurred among chicks fed such diets. Whitson et al. ('46b) and Bird et al. ('46) have reported that the addition of cow manure to breeder diets containing higher levels of soybean oil meal prevented the usual reduction in hatchability of eggs and viability of progeny.

The addition of methionine at levels up to 0.4% of the diet containing 70% soybean oil meal produced little or no increase in growth above that produced by the control diets. Several authors have shown that diets composed chiefly of corn and soybean oil meal can produce optimum growth when supplemented with methionine, yet methionine failed to overcome the detrimental effect of large quantities of soybean oil meal. This is evidence that the ill-effect of soybean oil meal

is not limited to interference with liberation of methionine in the digestive tract.

The fact that the growth factor of cow manure stored in the chick's body was able to counteract the effect of high soybean oil meal diets is additional evidence that the relationship involving the soybean oil meal, the growth factor and free methionine is not limited to the intestine.

It is also evident from the results of experiments 7 to 10 that the growth factor did not obviate the necessity of heat-treating soybean oil meal. Although the growth factor improved the nutritional value of raw soybean oil meal in an all-plant-protein diet, the use of heated soybean oil meal with the growth factor produced still better results.

From the evidence presented, it may be postulated that soybean oil meal contains an inhibitor of growth which is stable to heat, which produces an effect that is not confined to the digestive tract, and which, therefore, is not identical with the heat labile trypsin inhibitor reported by Ham and Sandstedt ('44) and crystallized by Kunitz ('46). The growth factor of cow manure has little or no effect upon the heat-labile inhibitor but counteracts the inhibiting effect which is not influenced by heat.

As an alternative to this hypothesis one might attempt to explain the differences in growth among groups fed 35, 50 and 70% soybean oil meal on the basis of the different quantities of barley and corn in the diets. It would be necessary to assume that these grains contain considerably more of the growth factor than does soybean meal. The report of Whitson et al. ('45) although it does not rule out this explanation completely, makes it difficult to believe that corn and barley contain significant quantities of the growth factor.

The distribution of the postulated heat-stable inhibitor in different samples of soybean products appears to be quite variable. Although no detailed study of this phase of the problem has been made, some samples of soybean oil meal have been obtained which produced optimum growth without a supplementary source of the growth factor when fed as

35% of the diet. Such meals have not been tried at the 70% level.

Further work is needed to determine if this growth inhibitor is confined to soybean products, and if the sole function of the growth factor of manure is to counteract this effect.

The results reported here would indicate that the benefits conferred by heating soybean oil meal and by supplementation with the growth factor are independent of each other. This subject should not be dismissed, however, without reference to the ability of methionine to act as a partial substitute for the growth factor (Bird et al., '47) and to the generally accepted role of this amino acid in the changes induced by heating soybean oil meal. According to Melnick et al. ('46) the poor utilization of raw soybean protein is due to inhibition of trypsin and consequent retarded liberation of methionine. Therefore, equally good results should be obtained with raw and with heated soybeans if each were supplemented with adequate methionine. Such results were not obtained either by Hayward and Hafner ('41) or by Almquist et al. ('42), perhaps because the highest levels of methionine they added to the raw soybean were inadequate or perhaps because the trypsin inhibitor disturbs protein digestion in other ways not involving the sulfur-containing amino acids. Heated soybean, because of the increased availability of its methionine, provides more of this amino acid for structural use and, if necessary, as a partial substitute for the growth factor than does the raw material. Even heated soybean oil meal, however, contains barely adequate, or slightly inadequate quantities, and so responds to methionine supplementation. It is supplemented more effectively by the growth factor.

Methionine appears, therefore, to be a connecting link between the trypsin inhibitor and the newly postulated, heat-stable growth-limiting influence. The trypsin inhibitor interferes with the liberation of methionine in the digestive tract and its effect is largely, if not entirely, negated by methionine feeding. The mechanism of the heat-stable inhibitor is unknown; its effect is counteracted partially and ir-

regularly by methionine but completely and consistently by the growth factor of cow manure.

Enzymatic treatment of raw soybean flour materially improved the nutritional value of this feedstuff. The hydrolysate did not quite equal the heated soybean oil meal, but in view of the entirely different origins of the 2 products, the results appear sufficiently similar to justify the statement that this experiment with enzyme-digested soybean flour confirmed the report by McGinnis and Menzies ('46) that papain digestion of raw soybean oil meal made it nutritionally equal to heated soybean oil meal. Hydrolysis, like heat-treatment, failed to overcome that portion of the growth inhibition which was counteracted by the growth factor of cow manure.

SUMMARY

Soybean oil meal fed to young chickens as 70% of the diet caused an inhibition of growth and increased mortality. Both of these effects were counteracted by the addition of the growth factor of cow manure to the diet but not by the addition of methionine.

The growth factor of cow manure improved the nutritional value of a chick diet containing raw soybean oil meal as the only protein concentrate, but not to the extent that it improved a diet containing heated soybean oil meal.

Evidence has been presented that soybean oil meal at high leve's exerts an inhibiting effect on the growth of chicks which is not due to a heat-labile trypsin inhibitor, which is not nullified by heating or by enzymatic digestion, but which is counteracted by the chick growth factor of cow manure.

LITERATURE CITED

ALMQUIST, H. J. 1946 Proteins and Amino Acids in Animal Nutrition. F. E. Booth Co., Inc., San Francisco.

ALMQUIST, H. J., E. MECCHI, F. H. KRATZER AND C. R. GRAU 1942 Soybean protein as a source of amino acids for the chick. J. Nutrition, *24:* 385.

BARNES, R. H., AND J. E. MAACK 1943 Review of the Literature on the Nutritive Value of Soybeans. The Hormel Institute of the University of Minnesota.

BIRD, H. R., AND J. P. MATTINGLY 1945 Addition of dl-methionine to starting and growing mashes. Poul. Sci., *24:* 29.

BIRD, H. R., M. RUBIN AND A. C. GROSCHKE 1947 An inborn characteristic determining the response of chickens to a diet free of animal protein. J. Nutrition, *33:* 319.

BIRD, H. R., M. RUBIN, D. WHITSON AND S. K. HAYNES 1946 Effectiveness of dietary supplements in increasing hatchability of eggs and viability of progeny of hens fed a diet containing a high level of soybean oil meal. Poul. Sci., *25:* 285.

EVANS, R. J., AND J. MCGINNIS 1946 The influence of autoclaving soybean oil meal on the availability of cystine and methionine for the chick. J. Nutrition, *31:* 449.

HAM, W. E., AND R. M. SANDSTEDT 1944 A proteolytic inhibiting substance in the extract from unheated soybean meal. J. Biol. Chem., *154:* 505.

HAYWARD, J. W., AND F. H. HAFNER 1941 The supplementary effect of cystine and methionine upon the protein of raw and cooked soybeans as determined with chicks and rats. Poul. Sci., *20:* 139.

KUNITZ, M. 1946 Crystalline soybean trypsin inhibitor. J. Gen. Physiol., *29:* 149.

MCGINNIS, J., AND J. H. MENZIES 1946 Effect of in vitro enzymatic digestion of raw soybean flakes on chick growth. Poul. Sci.,*25:* 538.

MELNICK, D., B. L. OSER AND S. WEISS 1946 Rate of enzymatic digestion of proteins as a factor in nutrition. Science, *103:* 326.

RUBIN, M., AND H. R. BIRD 1946a A chick growth factor in cow manure. I. Its non-identity with chick growth factors previously described. J. Biol. Chem., *163:* 387.

———— 1946b A chick growth factor in cow manure. II. The preparation of concentrates and the properties of the factor. J. Biol. Chem., *163:* 393.

WHITSON, D., J. C. HAMMOND, H. W. TITUS AND H. R. BIRD 1945 The use of soybean meal in the diet of growing chicks. II. The effect of different grains. Poul. Sci., *24:* 408.

WHITSON, D., H. W. TITUS AND H. R. BIRD 1946a Effect of dietary level of soybean meal on hatchability. Poul. Sci., *25:* 52.

———— 1946b The effect of feeding cow manure on egg production and hatchability. Poul. Sci., *25:* 143.

PROCEEDINGS OF THE ELEVENTH ANNUAL MEETING OF THE AMERICAN INSTITUTE OF NUTRITION

STEVENS AND CONGRESS HOTELS, CHICAGO, ILLINOIS, MAY 18–22, 1947

COUNCIL MEETINGS

Council meetings were held at the Stevens Hotel on Sunday, May 18. All members were present. Formal actions of the Council are reported in the minutes of the business section.

SCIENTIFIC SESSIONS

The scientific program consisted of 7 half-day sessions of scientific papers grouped according to topic. A total of 73 papers were presented and 9 were read by title. The abstracts of these papers were published in the Federation Proceedings, vol. 6, no. 1, March, 1947, pp. 401–424. If the number of papers increases much further it will be necessary to run simultaneous sections or to transfer papers to the list of those to be Read by Title.

BUSINESS SESSIONS

Two business meetings were held, one at 8:30 Monday evening, May 19, following the Institute Dinner, and the other at 4:00 P.M. Wednesday, May 21, following the scientific program. These meetings were presided over by the President, A. H. Smith.

Monday, May 19, 8:30 P.M. The report of the Treasurer was presented by E. M. Nelson. The Auditing Committee (Dr. Elsa Orent-Keiles and Dr. Chester Tolle) reported that the

Treasurer's books were in order. The Treasurer's report was approved.

Dr. George R. Cowgill, Editor of THE JOURNAL OF NUTRITION gave a brief report concerning the Journal as follows:

Beginning with the January 1946 issue a return was made to the page format that was used before the war. This change was made with all of the journals published by The Wistar Institute. As usual, 2 volumes were published during the year, namely volumes 31 and 32; they contained 118 articles. During the year 153 papers were submitted for consideration. The average number of papers per issue was 10, compared with 8.5 for the previous year. The average number of pages per article this year, namely 12.4, was higher than the previous year's figure of 9.0 pages. Simple comparison of these figures is not really valid because the format had been changed to the older one with fewer words per page; on the other hand it should be remembered that the number of pages per volume has been increased from 100 pages to the newer limit of 120.

Volume 31, covering January to June 1946, inclusive, was designated the "John R. Murlin Honor Volume" in honor of Dr. Murlin who founded the Journal and served as its editor until 1939 when he became an emeritus member of the American Institute of Nutrition. It was arranged that the first issue, that for January 1946, should carry a picture of Dr. Murlin which could serve as a frontispiece for the volume. The issue also carried a short note of appreciation of Dr. Murlin written by his younger colleague Dr. E. S. Nasset. A discussion of the efforts made to honor Dr. Murlin in some way, which culminated in the plan to designate volume 31 as an Honor Volume, was given in last year's report and therefore need not be given here (Fed. Proc., *5:* 316, Sept. '46).

The printing shop difficulties that made it impossible during the war years for our printer, The Wistar Institute, to bring out each issue on or about the tenth of each month were gradually overcome during the year. The most recent issues show a vast improvement in this regard. The one for April, 1947, was only a few days late; that for May, 1947, appeared

on schedule. In last year's report a hopeful statement was made regarding the prospects in this regard. Inasmuch as the latest issue appeared on time, we have every reason to believe that this problem caused by the war has now been solved, which is of course very gratifying to all parties concerned.

The members approved the Editor's Report and extended to Dr. Cowgill and the Editorial Board a hearty vote of thanks for their service.

The appointment of Dr. Walter C. Russell as the representative of the Institute to the Division of Biology and Agriculture of the National Research Council and as Liason Officer to the Food and Nutrition Board was approved. The appointment of Dr. L. A. Maynard as our representative on the Committee on International Congresses was approved.

The President reported that the Executive Committee of the Federation had discussed the feasibility of reorganizing the administration of the Federation by the establishment of a permanent business secretary who would, among other duties, be responsible for the annual meetings, thus removing much of this burden from the local committees. The Placement Service (which Dr. H. B. Lewis can no longer supervise) might also be transferred to the office of the permanent secretary. Such a proposal would require an increase of Institute dues to $4 ($3 to the Federation; $1 to the Institute). All of these recommendations were approved. The President also announced that the 1948 meeting would be held in Atlantic City.

It was voted that the Institute should have a dinner at the 1948 meeting.

Dr. Walter C. Russell, the Institute representative on the Committee on Organization of the American Institute of Biological Sciences, reported on the nature of the proposed new organization and indicated that the American Institute of Nutrition was eligible to become a charter member. After some discussion the invitation to join this new organization was declined on the basis that the Institute of Nutrition was

more closely related to the Federation Societies than to many of the proposed members of the American Institute of Biological Sciences.

The members observed a period of silence in honor of the following deceased members: Morris S. Fine, Charles L. Hoagland, Paul Roth, and Alfred Shohl.

Wednesday, May 21. The tellers reported that the following officers had been elected for 1947–1948:

President—R. M. Bethke
Vice-President—E. M. Nelson
Treasurer—N. R. Ellis
Councillor—A. D. Holmes
Associate Editors—E. W. Crampton
O. L. Kline
R. W. Swift

The following new members were elected on recommendation of the Council:

Aaron Arnold
J. C. Bauernfeind
Herbert R. Bird
Alex Black
David K. Bosshardt
George M. Briggs
W. W. Cravens
Eva Donelson
D. V. Frost
Ross A. Gortner
B. Connor Johnson
Seth Roth Johnson
Francis G. McDonald
Mary Elizabeth Reid
H. E. Robinson
Saul Rubin
H. P. Sarett
H. A. Schneider
Janice M. Smith
Clara A. Storvick
Madelyn Womack

The President announced that a Joint Committee on Nomenclature had been established in cooperation with the American Society of Biological Chemists. The committee consists of:

C. A. Elvehjem, Chairman
E. M. Nelson
A. D. Welch
H. J. Almquist

This action was approved.

Dr. Griffith presented to the Institute the essential content of bills currently under consideration by Congress (HR 3215 and Senate Bill 504) regarding the status of professional men

(other than physicians, dentists, and veterinarians) in the Medical Departments of the Army and Navy. These bills would group such professional men in a heterogeneous group of professional and non-professional workers (the Medical Service Corps). The defects of such a set-up were pointed out by Dr. Griffith. The Institute voted to send a protest to the House and the Senate regarding this situation and the President appointed a committee consisting of Drs. Griffith, Nasset, and Carter to frame appropriate letters. (These were sent to the chairmen of the House and Senate Committees on Armed Services on May 29.)

President Smith appointed the following Nominating Committee for 1947–1948:

Harold Goss, Chairman
H. G. Day
L. A. Maynard
H. B. Pierce
Pearl Swanson

The Institute gave a hearty vote of thanks to the Local Committee for their fine service in organizing and arranging the meeting.

The meeting adjourned at 4:45 P.M.

DINNER AND PRESENTATION OF AWARDS

For the first time since the war the Institute held a dinner meeting (Hotel Continental, May 19). Following the dinner Dr. Murlin gave a very interesting talk regarding the early history of the American Institute of Nutrition. At this time also the presentation of the Institute Awards was made. Dr. L. A. Maynard as recipient of the Borden Award received a medal and a check. Drs. Paul Day, W. J. Darby, and E. L. Stokstad as co-recipients of the Mead Johnson and Company Prize received scrolls and checks.

Respectfully submitted,

H. E. CARTER, *Secretary*
American Institute of Nutrition